# การพัฒนากระบวนการทางชีวภาพสำหรับการผลิต และคุณลักษณะเฉพาะของไฮโดรไลเสตคอลลาเจนเพพไทด์ ที่มีคุณสมบัติต้านอนุมูลอิสระจากกระดูกปลากดคัง

# Bioprocess Development for the Production and Characterisation of Hydrolysate Collagen Peptides with Antioxidant Properties from *Hemibagrus wyckioides* Bones

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## บทคัดย่อ

ปลากดคัง (*Hemibagrus wyckioides*) เป็นปลาน้ำจืดสายพันธุ์หนึ่งที่นิยมรับประทานในประเทศไทยโดยเฉพาะอย่างยิ่งส่วนของเนื้อปลา อย่างไรก็ตาม กระดูกปลากดคังเป็นวัตถุดิบเหลือทิ้งจากสถานประกอบการณ์ที่มีมูลค่าทางเศรษฐกิจต่ำ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาเปรียบเทียบวิธีการเตรียมไฮโดรไลเซทคอลลาเจนเพพไท์ในส่วนของร้อยละผลผลิต ร้อยละปริมาณโปรตีนรวม และเพื่อตรวจสอบคุณลักษณะและคุณสมบัติบางประการของไฮโดรไลเซทคอลลาเจนเพพไทด์จากกระดูกปลากดคัง ไฮโดรไลเซทคอลลาเจนเพพไทด์ถูกเตรียมขึ้นภายใต้ 5 สภาวะ ได้แก่ การใช้น้ำปราศจากไอออน (CLG-DI-24h, CLG-DI-48h), การใช้กรดอะซิติก (CLG-AC-24h, CLG-AC-48h), การย่อยด้วยเอนไซม์ปาเปน (CLG-PP-24h), การย่อยด้วยเอนไซม์อัลคาเลส (CLG-ACL-24h), และการใช้เอนไซม์เพพซินในกรดอะซิติก (CLG-ACP-24h, CLG-ACP-48h) ทุกสภาวะถูกบ่มที่อุณหภูมิ 37 °C ตัวอย่างในสภาวะกรดถูกสะเทิน กำจัดเกลือ ด้วยการไดอะไลซีส และทำให้แห้งด้วยเครื่องทำแห้งแช่เยือกแข็ง ตัวอย่างถูกวิเคราะห์หมู่ฟังก์ชันในโครงสร้างด้วยสเปกโทรสโกปีอินฟราเรดแบบฟูเรียร์ทรานสฟอร์มองค์ประกอบของกรดอะมิโนถูกศึกษาด้วยเทคนิคทินเลเยอร์โครมาโทกราฟฟี ร้อยละการส่องผ่านแสงของสารละลายตัวอย่างถูกวิเคราะห์ด้วยสเปคโตรโฟโตมิเตอร์ ซึ่งรวมถึงการประเมินคุณสมบัติการเกิดฟอง (การก่อตัวของฟองและความคงตัวของฟอง) การดูดกลืนแสงอัลตราไวโอเลต (ยูวี) ของสารละลายตัวอย่าง และกิจกรรมการกำจัดอนุมูลอิสระดีพีพีเอช ผลการศึกษาแสดงให้เห็นว่า CLG-ACP-24h มีร้อยละผลผลิตสูงสุด (98.77 ± 0.41%), ในขณะที่ CLG-ACL-24h ตรวจพบปริมาณโปรตีนรวมสูงที่สุด (98.43 ± 4.34%) เมื่อเทียบกับสภาวะการเตรียมอื่นๆ ทั้งหมด ($p < 0.05$) สเปคตรัมของสเปกโทรสโกปีอินฟราเรดแบบฟูเรียร์ทรานสฟอร์มถูกตรวจพบที่ตำแหน่ง 3400–3440 เซนติเมตร$^{-1}$ (เอไมด์ เอ), และ 1640–1655 เซนติเมตร$^{-1}$ (เอไมด์ 1) ซึ่งสอดคล้องกับการสั่นแบบยืดของพันธะ N–H และการสั่นแบบยืดของพันธะ C=O ตามลำดับ กรดอะมิโนที่ระบุได้ในตัวอย่างโดยวิธีโครมาโทกราฟฟีแบบกระดาษ ได้แก่ ไกลซีน โพรลีน และไฮดรอกซีโพรลีน มีค่าการเคลื่อนที่สัมพัทธ์ใกล้เคียงกับกรดอะมิโนมาตรฐาน CLG-AC-24h และ CLG-ACP-24h แสดงร้อยละของการส่องผ่านแสงในน้ำปราศจากไอออน (พีเอช 7.0) ที่ 95.97 ± 3.82, และ 94.80 ± 0.87 ตามลำดับ CLG-AC-24h และ CLG-ACP-24h แสดงคุณลักษณะของการเกิดโฟม โดยมีร้อยละความเสถียรภาพในการเกิดโฟม 100 ± 0.01 ที่ความเข้มข้น 20.0 มิลลิกรัมต่อมิลลิลิตร CLG-AC-24h และ CLG-ACP-24h แสดงค่าการดูดกลืนแสงสูงสุดที่ช่วง 190–230 นาโนเมตร ในขณะที่พบการ

ดูดกลืนแสงเพียงเล็กน้อยที่ช่วง 260–280 นาโนเมตร CLG-AC-24h และ CLG-ACP-24h มีฤทธิ์ต้านอนุมูลดีพีพีเอช เท่ากับ 32.95 ± 7.68 และ 31.03 ± 5.45% ตามลำดับ ผลการวิจัยเหล่านี้ชี้ให้เห็นว่ากระดูกของปลากดคัง ซึ่งเป็นผลพลอยได้จากอุตสาหกรรมอาจเป็นวัตถุดิบทางเลือกสำหรับการเตรียมไฮโดรไลเซทคอลลาเจนเพพไทด์ได้

**คำสำคัญ:** ปลากดคัง, ชีววัตถุ, การยับยั้ง, อาหารเพื่อสุขภาพ, ความปลอดภัย

## Abstract

The Asian red-tail catfish (*Hemibagrus wyckioides*) is a species of freshwater fish that is popular for consumption in Thailand, especially its flesh. However, *H. wyckioides* bones are waste materials from industrial processes with low economic value. This study aimed to compare methods for preparing hydrolysed collagen peptides from *H. wyckioides* bone in terms of yield and total protein content, and to examine their characteristic and functional properties. Hydrolysed collagen peptides were prepared under five conditions: deionised water (CLG-DI-24h and CLG-DI-48h), acetic acid (CLG-AC-24h and CLG-AC-48h), enzymatic digestion with papain (CLG-PP-24h), enzymatic digestion with Alcalase (CLG-ACL-24h), and enzymatic digestion with pepsin in acetic acid (CLG-ACP-24h and CLG-ACP-48h). All conditions were incubated at 37°C. The samples prepared under acidic conditions were neutralised, desalted by dialysis, and then freeze-dried. The samples were characterised for their functional groups by Fourier transform infrared (FTIR) spectroscopy. The amino acid composition was studied using thin-layer chromatography. The percentage transmittance (%T) of the sample solution was analyzed using a spectrophotometer. This included evaluating the foaming properties (foam formation and foam stability), the Ultraviolet (UV) absorption of the sample solutions, and the DPPH radical-scavenging activity. The results showed that CLG-ACP-24h exhibited the highest percentage yield (98.77 ± 0.41%), whereas CLG-ACL-24h exhibited the highest total protein content (98.43 ± 4.34%), among all the preparation conditions ($p < 0.05$). The FTIR spectra showed bands at 3400–3440 $cm^{-1}$ (amide A) and 1640–1655 $cm^{-1}$ (amide I), corresponding to N–H stretching and C=O stretching vibrations, respectively. The amino acids identified in the samples by paper chromatography, namely glycine, proline, and hydroxyproline, exhibited Retention factor ($R_f$) values similar to those of the standard amino acids. CLG-AC-24h and CLG-ACP-24h showed %T in deionized water (pH 7.0) of 95.97 ± 3.82, and 94.80 ± 0.87, respectively. CLG-AC-24h and CLG-ACP-24h exhibited foaming characteristics with foam stability of 100 ± 0.01% at a concentration of 20.0 mg/mL. CLG-AC-24h and CLG-ACP-24h showed a main absorbance peak at 190–230 nm, whereas only weak absorption was observed at 260–280 nm. CLG-AC-24h and CLG-ACP-24h exhibited DPPH radical scavenging activity of 32.95 ± 7.68 and 31.03 ± 5.45%, respectively. These findings suggest that *H. wyckioides* bone, an industrial by-product, may be an alternative raw material for the preparation of hydrolysate collagen peptides.

**Keywords:** Red-tailed mystus fish, Biomaterial, Inhibition, Functional food, Safety

## 1. Introduction

Collagens are the fibrous proteins localized in the extracellular matrix of connective tissue, including vertebrate tendons, cartilage, and bones. They display the physiological function to support the stability of cell structures. There have been reports of several collagens with animal origins, ranging from Type I to XXVIII (Karsdal, 2023). Approximately 30 % of all animal proteins are found to be collagens (Birk & Bruckner, 2005). Collagen type I is the most dominant collagen, with 90% of all collagen types. Collagen's wide-ranging uses in cosmetics, food processing, and biomedical fields are driving strong global

demand. As a result, the market was forecasted to nearly double from US$3.61 billion in 2016 to US$6.63 billion in 2025 (Orel et al., 2021; Wang, 2021). Previously, pigs and cows were considered the primary sources of raw materials used to produce collagen (Ogawa et al., 2004; Orel et al., 2021). However, the use of pigs and cows in collagen production is associated with several concerns: (1) religious restrictions (Islam, Buddhism, Hinduism, and Judaism); (2) the risk of transmitting zoonotic diseases, such as foot-and-mouth disease (FMD), bovine spongiform encephalopathy (BSE), and transmissible spongiform encephalopathy (TSE); and (3) the potential for allergic reactions (Orel et al., 2021; Wang, 2021). Therefore, new sources of collagen are being investigated.

Aquatic animals are an alternative source for gathering collagen. The freshwater and marine organism industries produce a high number of processed byproducts, remarkably the fish processing byproducts such as scales, skin, bones, and fins (Zhang et al., 2011; Chalamaiah et al., 2012; Nguyen & Thai, 2024). These sources are used for the preparation of bioactive peptides, including hydrolysate collagens (Ishak & Sarbon, 2018; Abdo et al., 2023).

Improving the functional properties of proteins can broaden their range of applications. The structure of proteins can be modified through several methods, including physical (heat, high-pressure processing, ultrasound), chemical (acidic, alkaline, or saline), and biological (Jafari et al., 2020; Nguyen & Thai, 2024; Kaya et al., 2025). The preparation of hydrolysed peptides using chemical methods, such as sulfuric acid, causes an unpleasant odour (Kristinsson & Rasco, 2000; Pasupuleti & Demaimay, 2010). Additionally, hydrochloric acid causes the loss of the amino acids tryptophan, cysteine, serine, and methionine (Fountoulakis & Lahm, 1998). Protein breakdown by alkali (alkali hydrolysis) can cause some amino acids to undergo racemisation, converting L-amino acids into D-amino acids, which cannot be utilised by the body. In addition, this process degrades certain amino acids, such as arginine, threonine, and cysteine (Friedman, 1999). Owing to these limitations, chemical preparation methods are not favoured in the food industry. A widely used method is protease hydrolysis, because the enzyme exhibits high substrate specificity and efficiently accelerates the breakdown of peptide bonds (Kadam & Aluko, 2026).

Generally, several process factors must be considered, including the enzyme-to-substrate ratio, reaction duration, pH, and temperature. Therefore, controlling the optimal conditions for protease hydrolysis affects the characteristics of the resulting hydrolysed peptide product (Jafari et al., 2020; Kaya et al., 2025). However, the main limitation of using enzymes is the high cost. Additionally, enzymes can naturally degrade, which can lead to reduced biological activity. One possible solution is a hybrid or integrated approach that combines chemical and biological (enzymatic) methods, which could help address these limitations.

Red-tailed Mystus (*Hemibagrus wyckioides*), or red-tailed catfish, is a fish species in the family Bagridae. The natural freshwater rivers are the habitat of *H. wyckioides*. The original distribution of *H. wyckioides* was found along the Mekong River. With a body length of 130 cm and a weight of 80 kg, the red-tail catfish is the largest member of the Bagridae family (Shao et al., 2021). *H. wyckioides* is a freshwater fish species popular for consumption owing to its weaker fishy and muddy odour, flavourful flesh, and high protein content. Moreover, *H. wyckioides* fish also exhibits strong disease resistance, rapid growth, and ease of domestication, which contribute to its increased economic value (Shao et al., 2021; Zhou et al., 2023). Based on the survey data on aquatic resources in Thailand, compiled by the Fisheries Development Policy and Planning Division (2019) for the year 2019, statistics on freshwater aquaculture production were collected. The data indicate that fish constitute the largest group of aquatic organisms of economic importance, with Nile tilapia and catfish ranking as the top two species. According to the findings, freshwater fish categorised under “other species” accounted for 4.26% of total production. This group generated

economic value amounting to 899,669,000 baht from the total volume of all freshwater aquatic organisms. Among these, the *H. wyckioides* represents a significant contributor to economic value. The total volume of *H. wyckioides* reached 1,403 tonnes, with an aggregate market value of 144,646,000 baht. With the increase in farming, consumer demand, and the expansion of the processing industry, the number of by-products from *H. wyckioides* has also increased (Shao et al., 2021; Zhou et al., 2023).

However, studies on value-added utilisation of by-products from *H. wyckioides* fish remain limited, especially data on the preparation of hydrolysed collagen peptides. Therefore, this study aimed to compare three methods, chemical, biological (enzymatic), and a combination of both, for preparing hydrolysed collagen peptides from *H. wyckioides* bone, and to examine their characteristics and functional properties. The results of this study provide a method for preparing hydrolysed collagen peptides from *H. wyckioides* bone, which possess several important biological and functional properties. These findings may contribute to zero waste in the *H. wyckioides* processing industry and support the value-added utilisation of this by-product.

## 2. Materials and methods

The experimental protocols used in this study were reviewed and approved by the Research Ethics Committee of Rajamangala University of Technology Thanyaburi (Approval No. RMUTT.SCI.2022.R009).

### 2.1 Materials and Chemicals

Nhan Tailed Mystus fish entrepreneur in Bangplee district, Samut Prakan province, Thailand, purchased fresh *H. wyckioides* (Figure 1) from a Tantarum fish farm in Bangbao district, Samut Prakan province, Thailand. This farm received the Certificate of Good Aquaculture Practice (GAP) from the Department of Fisheries, Thailand. Glacial acetic acid, *n*-butanol, sodium hydroxide (NaOH), trichloroacetic acid (TCA), and ethanol were obtained from RCI Labscan Limited (Thailand). The standard amino acids and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and alcalase were purchased from HiMedia Laboratories Private Limited (India) and Sigma Aldrich. Standard collagen peptides, Type I and II, were bought from Nippi Peptide FCP-EX-G (Japan) and 10KATE laboratory (Netherlands), respectively. Pepsin (activity, 3000NF) and papain powder were ordered from Xi'an Pincredit Bio-Tech Co., Ltd (China).



**Figure 1.** Representative of 10 months red-tailed Mystus fish (*H. wyckioides*) from Tantarum fish farm in Bangbao district, Samut Prakan province, Thailand. *H. wyckioides* is approximately 8–10 months of age. The fish average weight of 1-1.2 kilograms each, which is the size at which they are sold to the processing industry and restaurant businesses

### 2.2 *H. wyckioides* bone preparation

In this research, *H. wyckioides* bones were supported by Nhan Tailed Mystus fish entrepreneur in the amount of 50 kg. The fresh *H. wyckioides* bones were placed in an ice box to be transferred to the laboratory. The bones were cut to the same size (2-5 cm). To remove the earthy odour, *H. wyckioides* bones were weighed, and then placed into the chambers placed/ immersed/ submerged in ice-cold deionized water (DI). The chambers containing *H. wyckioides* bones were sonicated using the 35 kHz normal attitude ultrasonic bath (Sonorex digitec, Bandelin, Germany) at 4 °C, for 5 min, three times. The water was transferred. Next, to remove the musty off-flavors, the bones were washed with 5% v/v of ice-cold acetic acid at a ratio of 1:2 w/v. Then, the containers were sonicated using the same conditions. In the last step, the bones were washed with ice-cold DI water, like the above technique. To enhance collagen purity, non-collagenous proteins were removed using a method adapted from Chuaychan et al., (2015). The deodorised bones of *H. wyckioides* were placed into the chambers containing ice-cold sterile 0.1 M NaOH at 1:10 w/v. The containers were incubated at 4 °C for 2 h and this process was repeated three times. The deproteinised bones were washed with ice-cold sterile DI water to neutral (pH 7.0). The nearly dry bones were homogenised using a homogeniser at 10,000 rpm, 4 °C for 30 seconds. The homogenised bones were equilibrated for use in the next procedure.

### 2.3 Collagen extraction process

Generally, there are various processes for extracting active peptides from fish waste components, including acid, alkaline, and enzyme extraction under multiple conditions. In this study, collagen-derived peptides from *H. wyckioides* bone were prepared using a method adapted by combining approaches from several previous studies (Naqash & Nazeer, 2011; Jafari et al., 2020; Kaya et al., 2025).

In the initial step**,** the 20 g of homogenate bones (dried weight average 6.22 g) was divided into 8 groups, with 3 replicates per group. For the control group, 200 mL of ice-cold sterile DI water was placed into an extraction chamber and then was shaken at 120 rpm and incubated at 37°C for 24–48 h. The reaction was then terminated by immersing the extraction chambers in boiling water at 100°C for 5 min. The samples were collected and freeze-dried using a lyophiliser (OPERON, South Korea). In addition, 200 mL of 0.5 M sterile acetic acid and 1.0% w/v pepsin in 0.5 M acetic acid were added to the extraction chambers for the chemical and combined chemical-biological methods, respectively. Then, the reactions were incubated and shaken at the same temperature, speed, and duration as described for the control group. However, the reaction in these chambers were stopped by titration with 10% w/v NaOH. The supernatant of these conditions was collected and desalted by a 3.5 kDa cut-off dialysis bag in 6-fold ice-cold sterile double-distilled water for four times. Then, the desalted supernatants of each condition were frozen dry.

Moreover, for the biological method, 1.0% w/v papain and 1.0% w/v alcalase were added to each chamber in the same volume as the above conditions. These extraction chambers were treated under the same conditions as the control group, except that the incubation time was only 24 h. The reactions for the papain and alcalase conditions were terminated by immersing in boiling water at 100°C for 5 min. The supernatant from each condition was collected and dried using lyophilizer. The dried powder of the hydrolysed collagen peptides was weighed to calculate the percentage yield, and the total protein content was determined using the Kjeldahl method (AOAC, 2023). Dry weight, percentage sample recovery, and the total protein contents under all conditions were statistically compared at a significance level of $p < 0.05$.

### 2.4 Determination of functional groups using Fourier transform infrared spectrum (FTIR)

Functional group analysis of the samples was performed by FTIR according to the standard in-house method specified in the instrument's operating manual. Standard potassium bromide (KBr) was

accurately weighed, finely ground using a mortar and pestle, and oven-dried to remove residual moisture. A mass of 0.1 g of the dried KBr powder was immediately transferred to a desiccator. The powder was then placed into a sample holder and compressed using a hydraulic press at a pressure of 10 MPa for 5 min to form a KBr pellet. The prepared KBr pellet was stored in a desiccator prior to analysis. Approximately 0.001–0.005 g of the dried sample powder was thoroughly mixed with the KBr powder. The resulting mixture was processed following the same procedure as that used for the standard KBr pellet preparation. The sample–KBr pellet was subsequently analysed by Fourier-transform infrared (FTIR) spectroscopy (SPECTRUM ONE, PERKIN ELMER, USA) in the spectral region of 400-4000 $cm^{-1}$ (Yang et al., 2021), with an absolute threshold of 0.0003 and a sensitivity of 37. The percentage transmittance was then recorded.

**2.5 Determination of amino acids of digested collagen peptides using Thin-layer chromatography (TLC)**

The dried powder of CLG-STD-II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h were collected at 0.01 g and placed into sterile microtubes. Then, 100 µL of 6 M hydrochloric acid (HCl) was added into microtubes, mixed vigorously, and incubated at 100°C for 120 h. The reactions were centrifuged at 3000 rpm for 5 min. The clear supernatant was collected and neutralized for separation by paper chromatography. The 0.01 g of the standard amino acids (glycine, proline, hydroxyproline, lysine, alanine, leucine, and isoleucine) was dissolved in DI to 10 mg/mL. All standard solutions and supernatants of each sample were spotted on the TLC sheet. The stationary phase was placed into the separating chamber that contained the mobile phase (n-butanol: acetic acid: water, 3:1:1). The chamber was closed and incubated until the mobile phase moved to the ending line. The stationary phase was oven to 100°C for 10 min. The 1.0% w/v of ninhydrin in ethanol was sprayed on the stationary phase and oven to 100°C for 10 min. The retention factor ($R_f$) was analyzed according to the formula below.

$$R_f = X/Y \quad (1)$$

X = The distance ending of sample spot (cm)
Y = The distance ending of the mobile phase (cm)

**2.6 Investigation percentage transmittance (%T) of dissolved collagen hydrolysate peptides**

The powder of CLG-STD-II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h was placed to tubes and then dissolved by deionized water (DI) and 5.0% w/v trichloroacetic acid (5% w/v TCA) to a concentration of 250 µg/mL. The mixture in each tube was vortexed gently for 5 min. The dissolved samples were measured for % transmittance at the wavelength 600 nm using a UV-Vis spectrophotometer (Thermo Scientific, Sweden).

**2.7 UV-Vis Absorption Analysis**

The purity of hydrolysate collagen peptides was modified according to a previous report by Zhang et al., (2011). The 0.001g of CLG-STD-II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h powder was dissolved in DI water for 1.0 mg/mL preparation. The solution was placed into a quartz cell to determine the absorbance values at wavelengths of 190–340 nm using the scanning mode of a UV-visible spectrophotometer (FP-8500, JASCO, JAPAN), following a slightly modified method from Kaya et al., (2025). The data was recorded and plotted in the graphs. The wavelength was set to the *X*-axis and paired with the *Y*-axis as the absorbance values.

### 2.8 Foam formation and foam stability assay

A slightly modified version of the procedure outlined by Naghdi et al., (2023) was used to determine the foaming capacity and stability of the hydrolysed collagen peptides. The dried powder of CLG-STD-II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h was dissolved in DI. The samples were prepared to 5, 10, and 20 mg/mL for 20 mL. The 20 mL of sample solution was investigated, and the pH value was then placed into the 50 mL cylinder. The initial volume ($V_i$) was recorded. The homogenizer probe was positioned into a cylinder and localized at a height of $V_i$. The homogenizer was turned on to produce the foaming for 1 min. The volume after homogenization was read and recorded as $V_1$. The reaction was allowed to stand at room temperature for 30 min, and then the foaming ($V_{30}$) volume was recorded. Foaming and stability were calculated following the equation below:

$$\text{Foam expansion (\%)} = [(V_1-V_i)/V_i] \times 100 \quad (2)$$

$$\text{Foam stability (\%)} = [(V_{30}-V_i)/(V_1-V_i)] \times 100 \quad (3)$$

### 2.9 Antioxidant activity assay

This study used a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay to determine the antioxidant potential of collagen and collagen peptides. This experiment was briefly modified from the previously described one by Patathananone et al., (2019). The DPPH powder was dissolved in ethanol to a concentration of 0.2028 mM. This solution was diluted two-fold using DI water. The 190 μL of 0.1014 mM DPPH solution (0.1014 mM DPPH in 50% v/v ethanol) was subjected to wells of the 96-well plate. A 10 μL aliquot of each sample solution (at an initial concentration of 500 μg/mL) was added to the DPPH solution. The plate was incubated at 37°C in the dark for 30 min. The absorbance values were detected at a wavelength of 515 nm using a microplate reader (EZ-Read 2000, Biochrom, United Kingdom). The percentage of antioxidant activity was evaluated following the formula below.

$$\text{\% Inhibition} = \frac{[Ab_{515\ control} - blank_{control}] - [Ab_{515test} - blank_{test}] \times 100}{[Ab_{515\ control} - blank_{control}]} \quad (4)$$

### 2.10 Statistical analysis

The dry weight, percentage yield, total protein contents, percentage transmittance (%T), foam formation, and foam stability of the hydrolysate collagen peptides were calculated and are presented as mean ± SD (n = 3). One-way ANOVA was used to evaluate statistical differences among treatments, and Duncan's Multiple Range Test (DMRT) was applied for post hoc comparisons. Values marked with different superscript letters differ significantly ($p < 0.05$).

## 3. Results

### 3.1 Dried weight, yield, and total protein contents

The preparation of hydrolysate collagen peptides derived from the bones of *H. wyckioides* is presented in Figure 2 and Table 1. Three extraction approaches were evaluated: (1) chemical (CLG-AC-24h and CLG-AC-48h); (2) biological (CLG-PP-24h and CLG-ACL-24h), and (3) combined chemical–biological (CLG-ACP-24h and CLG-ACP-48h), with deionised water (CLG-DI-24h and CLG-DI-48h) as the control group.

The physical appearance of the products differed depending on the extraction method. CLG-DI-24h and CLG-ACL-24h appeared as viscous, gelatin-like solids, whereas CLG-PP-24h formed a light cream-coloured powder. Both CLG-AC-24h and CLG-ACP-24h appeared as white powders.

After 24 h of incubation, the dry weights of CLG-AC-24h, CLG-PP-24h, CLG-ACL-24h, and CLG-ACP-24h were 5.88 ± 0.07, 5.58 ± 0.14, 5.16 ± 0.03, and 6.14 ± 0.03 g, respectively, with significant differences observed among the treatments ($p < 0.05$). In addition, CLG-DI-24h and CLG-DI-48h showed dry weights of 4.24 ± 0.09 and 4.74 ± 0.06 g, respectively, which were significantly lower than those of the samples prepared by all three other methods at 24 h ($p < 0.05$). However, CLG-AC-48h and CLG-ACP-48h were found to have dry weights of 1.13 ± 0.02 g and 2.09 ± 0.07 g, respectively, which were significantly lower than those of all the conditions mentioned above ($p < 0.05$).

Moreover, CLG-DI-24h, CLG-DI-48h, CLG-AC-24h, CLG-AC-48, CLG-PP-24h, CLG-ACL-24h, CLG-ACP-24h, and CLG-ACP-48h exhibited the percentage yield (% yield) of 68.22 ± 2, 76.15 ± 1.16, 94.48 ± 1.11, 18.22 ± 1.35, 89.76 ± 2.42, 83.01 ± 0.49, 98.77 ± 0.41, and 33.55 ± 0.41%, respectively. Among all treatments, CLG-ACP-24h showed the highest percentage yield (98.77 ± 0.41%) ($p < 0.05$).

However, the total protein content varied markedly among treatments. CLG-ACL-24h (98.43 ± 4.34%) and CLG-PP-24h (84.20 ± 9.72%) exhibited substantially higher protein content than CLG-AC-24h (32.24 ± 0.19%) and CLG-ACP-24h (33.77 ± 0.03%). The control samples (CLG-DI-24h and CLG-DI-48h) showed the lowest values.




**Figure 2.** The morphology of protein/peptide products extracted by deionised water, acidic reagents, and protease enzymes. (A) CLG-DI-24h, (B) CLG-AC-24h, (C) CLG-ACL-24h, (D) CLG-PP-24h and (E) CLG-ACP-24h. CLG-DI-24h, CLG-AC-24h, CLG-ACL-24h, CLG-PP-24h, and CLG-ACP-24h are the ID name of hydrolysate collagen peptides that was prepared by 6.22 g of the dried powder homogenate bones, incubated in DI water (A), 0.5 M acetic acid (B), 1.0% w/v alcalase (C), 1.0% w/v papain (D), and 1.0% w/v pepsin in 0.5 M acetic acid (E), respectively. All conditions were shaken at 120-160 rpm, 37°C for 24 h, and frozen dry to powder using lyophilization

**Table 1.** Dry weight of hydrolysate peptides yield and total proteins in each extracted condition

| Conditions | Extracted conditions | ID Names | The dry weight of collagen hydrolysate peptides (g) | Yield (%) | Total proteins (%) |
|---|---|---|---|---|---|
| Control | (1) DI | CLG-DI-24h | 4.24 ± 0.09$^{f}$ | 68.22 ± 2.00$^{f}$ | 7.00 ± 3.30$^{a}$ |
| | | CLG-DI-48h | 4.74 ± 0.06$^{e}$ | 76.15 ± 1.16$^{e}$ | 9.56 ± 3.98$^{a}$ |
| Acidic (Chemical) | (2) 0.5 M acetic acid | CLG-AC-24h | 5.88 ± 0.07*$^{b}$ | 94.48 ± 1.11$^{b}$ | 32.24 ± 0.19$^{b}$ |
| | | CLG-AC-48h | 1.13 ± 0.02*$^{h}$ | 18.22 ± 1.35$^{h}$ | - |
| Enzymatic (Biological) | (3) 1.0% w/v papain | CLG-PP-24h | 5.58 ± 0.14$^{c}$ | 89.76 ± 2.42$^{c}$ | 84.20 ± 9.72$^{c}$ |
| | (4) 1.0% w/v alcalase | CLG-ACL-24h | 5.16 ± 0.03$^{d}$ | 83.01 ± 0.49$^{d}$ | 98.43 ± 4.34$^{d}$ |
| Enzymatic with acidic (Chemical + Biological) | (5) 1.0% w/v pepsin in 0.5 M acetic acid | CLG-ACP-24h | 6.14 ± 0.03*$^{a}$ | 98.77 ± 0.41$^{a}$ | 33.77 ± 0.03$^{b}$ |
| | | CLG-ACP-48h | 2.09 ± 0.07*$^{g}$ | 33.55 ± 0.41$^{g}$ | - |

**Note:** The dried weight of hydrolysate collagen peptides, percentage yield, and total proteins are represented as mean ± SD. The statistical differences between groups were analysed using one-way ANOVA. The superscript letters (a-h) showed statistically significant differences at $p < 0.05$. CLG-DI-24h, CLG-DI-48h, CLG-AC-24h, CLG-AC-48h, CLG-PP-24h, CLG-ACL-24h, CLG-ACP-24h, and CLG-ACP-48h are the ID names of hydrolysate collagen peptides. The conditions of each ID name were expressed below:

(1) CLG-DI-24h and CLG-DI-48h: Died weight of homogenate bones (6.22 g) were incubated in DI water, shaked 120-160 rpm at 37°C for 24 and 48 h

(2) CLG-AC-24h and CLG-AC-48h: Died weight of homogenate bones were incubated in 0.5 M acetic acid, shaked 120-160 rpm at 37°C for 24 and 48 h

(3) CLG-PP-24h: Died weight of homogenate bones (6.22 g) were incubated in 1.0% w/v papain, shaked 120-160 rpm at 37°C for 24 h

(4) CLG-ACL-24h: Died weight of homogenate bones (6.22 g) were incubated in 1.0% w/v alcalase, shaked 120-160 rpm at 37°C for 24 h

(5) CLG-ACP-24h and CLG-ACP-48h: Died weight of homogenate bones (6.22 g) were incubated in 1.0% w/v pepsin in 0.5 M acetic acid, shaked 120-160 rpm at 37°C for 24 and 48 h

### 3.2 FTIR spectra of hydrolysate collagen peptides

The FTIR spectra of STD-CLG-II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h are shown in Figure 3A-D. The results showed that the FTIR spectrum of CLG-STD-II (Figure 3A) exhibited bands at 579, 920, 1027, 1242, 1405, 1653, 2924, and 3402 $cm^{-1}$. The FTIR spectrum of CLG-DI-24h (Figure 3B) presented bands at 727, 1238, 1465, 1655, 1745, 2925, and 3397 $cm^{-1}$. The FTIR spectrum of CLG-AC-24h (Figure 3C) exhibited bands at 809, 1020, 1416, 1561, 1646, and 3457 $cm^{-1}$. Similarly, the FTIR spectrum of CLG-ACP-24h (Figure 3D) showed bands at 809, 1020, 1413, 1561, 1643, 1703, and 3433 $cm^{-1}$. An IR peak at around 3397-3457 $cm^{-1}$ was found in all three prepared samples and matched the band observed in standard collagen type II (STD-CLG-II). The band appearing at 3400–3440 $cm^{-1}$ has been reported as the amide A band, corresponding to N–H stretching vibrations. The amide I band (1640–1655 $cm^{-1}$), associated with C=O stretching, was present in all samples. The C–H stretching band at 2924–2933 $cm^{-1}$ was clearly observed in STD-CLG-II and CLG-DI-24h but appeared weaker in CLG-AC-24h and CLG-ACP-24h. In addition, the wavenumber range of 1230–1245 $cm^{-1}$, associated with C–N and N–H bend, was observed in STD-CLG-II and CLG-DI-24h but was absent from CLG-AC-24h and CLG-ACP-24h.

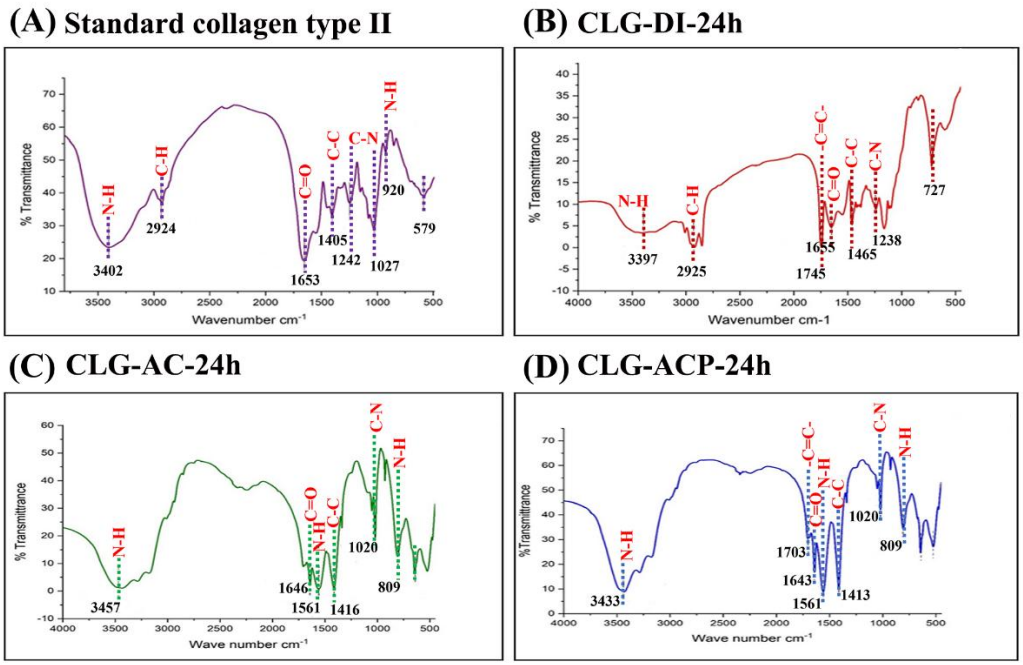



**Figure 3.** The FTIR spectrum of samples. (A) Standard collagen type II (STD-CLG-II), (B) CLG-DI-24h, (C) CLG-AC-24h, and (D) CLG-ACP-24h. CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h are the ID names of collagen hydrolysate peptides that were prepared by 6.22 g of the dried powder homogenate bones, incubated in DI water, 0.5 M acetic acid, and 1.0% w/v pepsin, respectively. All conditions were shaken at 120-160 rpm, 37°C for 24 h

### 3.3 Amino acid composition by thin-layer chromatography

The chromatographic profiles of amino acids separated by thin-layer chromatography (TLC) are presented in Figure 4. In this study, lysine, glycine, hydroxyproline, alanine, proline, isoleucine, and leucine were used as standard amino acids to compare their $R_f$ values with those of the spots found in the hydrolysed collagen peptides prepared from *H. wyckioides* bones. These standard amino acids constitute a major part of the amino acid composition of collagen from various sources and are present in relatively high proportions (Duan et al., 2009). The $R_f$ values of the standard amino acids compared with those of the amino acids in the samples are shown in Table 2. Lysine, glycine, hydroxyproline, alanine, proline, isoleucine, and leucine, exhibited $R_f$ values of 0.22, 0.27, 0.29, 0.38, 0.38, 0.64, and 0.67, respectively. CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h showed spots with $R_f$ values close to those of several standard amino acids, including glycine, hydroxyproline, proline, alanine, isoleucine, and leucine. However, the analysis in this study was qualitative in nature, providing preliminary data for the subsequent quantitative analysis of amino acid composition using advanced techniques.

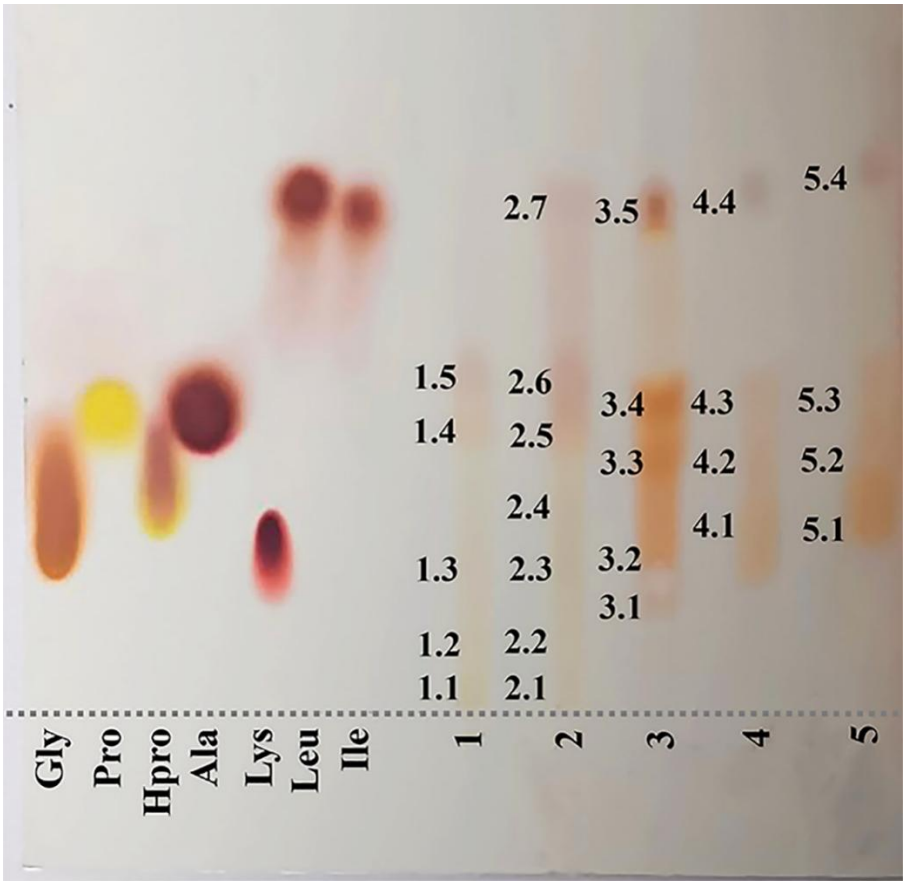



**Figure 4.** The standard amino acids and samples separated profile on TLC. The standard amino acids are glycine (Gly), proline (Pro), hydroxyproline (Hpro), alanine (Ala), lysine (Lys), leucine (Leu), and Isoleucine (Ile). Lanes 1, 2, 3, 4, and 5 showed the separation profile of acid hydrolysate of standard collagen type I (STD-CLG-I), standard collagen type II (STD-CLG-II), CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h, respectively. The $R_f$ of each spot number is represented in Table 2

**Table 2**. Retention factor ($R_f$) of standard amino acid and the amino acid products from hydrolysate peptides using strong acid reagent

| Retention factor ($R_f$) | | | | | |
|---|---|---|---|---|---|
| STD amino acids | Collagen Type I | Collagen Type 2 | CLG-DI-24h | CLG-AC-24h | CLG-ACP-24h |
| Lys = 0.22 | Spot 1.1 = 0.04 | Spot 2.1 = 0.04 | Spot 3.1 = 0.15 | Spot 4.1 = 0.24 | Spot 5.1 = 0.24 |
| Gly = 0.27 | Spot 1.2 = 0.10 | Spot 2.2 = 0.10 | Spot 3.2 = 0.20 | Spot 4.2 = 0.32 | Spot 5.2 = 0.32 |
| Hpro = 0.29 | Spot 1.3 = 0.18 | Spot 2.3 = 0.18 | Spot 3.3 = 0.32 | Spot 4.3 = 0.39 | Spot 5.3 = 0.41 |
| Ala = 0.38 | Spot 1.4 = 0.37 | Spot 2.4 = 0.26 | Spot 3.4 = 0.39 | Spot 4.4 = 0.64 | Spot 5.4 = 0.67 |
| Pro = 0.38 | Spot 1.5 = 0.42 | Spot 2.5 = 0.29 | Spot 3.5 = 0.64 | | |
| Ile = 0.64 | | Spot 2.6 = 0.42 | | | |
| Leu = 0.67 | | Spot 2.7 = 0.64 | | | |

**Note:** CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h are the ID names of hydrolysate collagen peptides that were prepared by 6.22 g of the dried powder homogenate bones, incubated in DI water, 0.5 M acetic acid, and 1.0% w/v pepsin, respectively. All conditions were shaken at 120-160 rpm and 37°C for 24 h.

### 3.4 Percentage transmittance (%T)

The percentage transmittance data of the hydrolysed collagen peptides and standard collagen type II (STD-CLG-II) in solvents with different pH levels are shown in Figure 5. The results indicated that at 250 μg/mL, STD-CLG-II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h dissolved in deionized (DI) water (pH 7.0) exhibited percentage transmittance (%T) values of 63.90 ± 0.52, 64.37 ± 2.12, 95.97 ± 3.82, and 94.80 ± 0.87, respectively. In addition, when dissolved in an acidic solvent (5% w/v TCA), STD-CLG-II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h exhibited %T values of 58.83 ± 0.81, 82.20 ± 0.72, 97.60 ± 1.42, and 93.63 ± 3.03, respectively. CLG-AC-24h and CLG-ACP-24h exhibited significantly higher %T values than those of STD-CLG-II and CLG-DI-24h ($p < 0.05$). Furthermore, the %T of STD-CLG-II in water was higher than that in the acid solvent, whereas CLG-DI-24h showed a higher %T in the acid solvent than in water; both differences were statistically significant ($p < 0.05$).

### 3.5 UV–Vis spectrophotometric data

The ultraviolet absorption of the sample solutions was analysed at wavelengths of 190–340 nm to examine the amino acids containing aromatic rings in their side chains, namely phenylalanine, tyrosine, and tryptophan. The results are shown in Figure 6A-F. The results showed that phenylalanine (Figure 6A), tyrosine (Figure 6B), and standard collagen type II (STD-CLG-II) (Figure 6C) had two main absorbance peaks, at 190–230 nm and 260–280 nm. On the other hand, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h showed a main absorbance peak at 190–230 nm, with a smaller peak at 260–280 nm. This indicates that the hydrolysed collagen peptides had a low signal from aromatic amino acids.

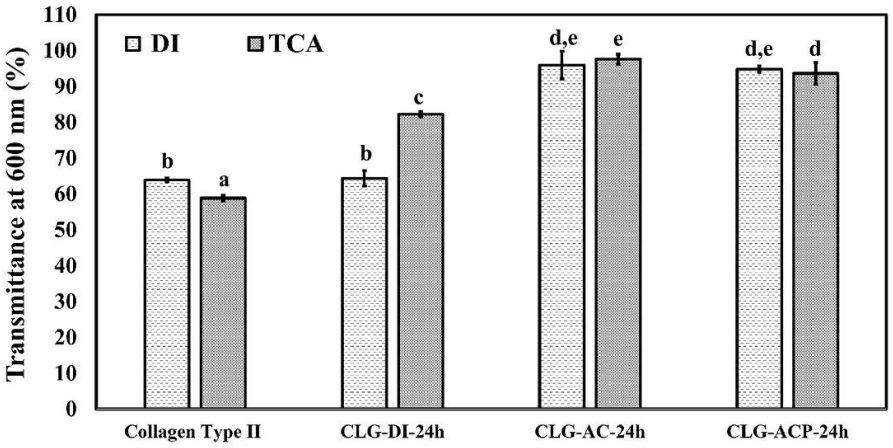



**Figure 5.** The percentage transmittance (%T) of sample solution that was dissolved in deionised water (DI) and 5.0% w/v trichloroacetic acid (5.0% w/v TCA). The 250 μg/mL was the final concentration of each condition. CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h are the ID names of collagen hydrolysate peptides that were prepared by 6.22 g of the dried powder homogenate bones, incubated in DI water, 0.5 M acetic acid, and 1.0% w/v pepsin, respectively. All conditions were shaken at 120-160 rpm, 37°C for 24 h. The %T represented as mean ± SD (n = 3). The statistical differences between groups were analysed using one-way ANOVA. The superscript letters (a-e) showed statistically significant differences at $p < 0.05$.

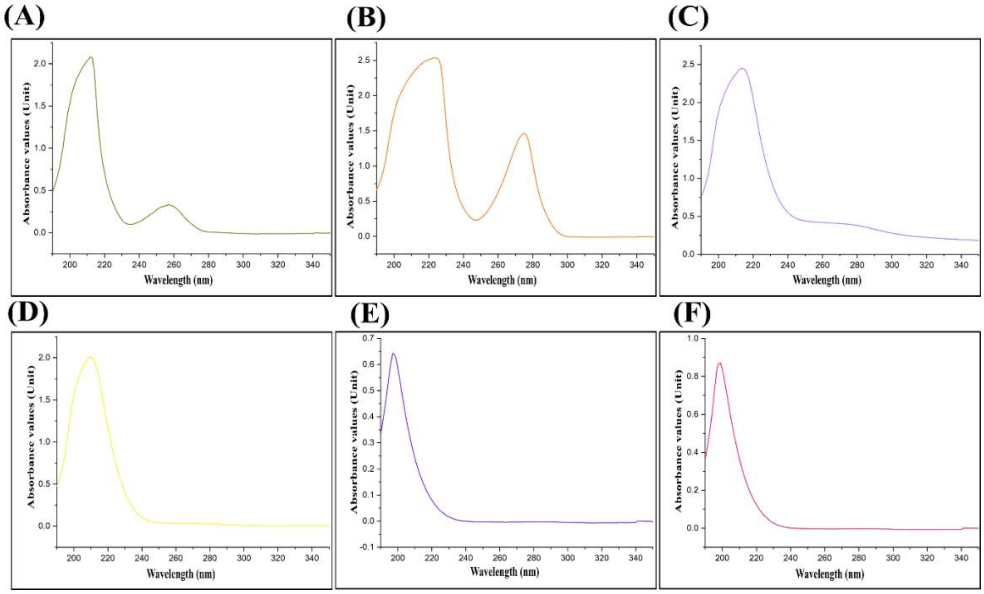



**Figure 6.** The scanning absorbance values profile of 250 μg/mL sample solutions at UV wavelength 190 - 340 nm. (A) phenylalanine, (B) Tyrosine, (C) Standard collagen type II (STD-CLG-II), (D) CLG-DI-24h, (E) CLG-AC-24h, and (F) CLG-ACP-24h. CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h are the ID names of hydrolysate collagen peptides that were prepared by 6.22 g of the dried powder homogenate bones, incubated in DI water, 0.5 M acetic acid, and 1.0% w/v pepsin, respectively. All conditions were shaken at 120-160 rpm, 37°C for 24 h.

### 3.6 Foaming properties

The results of the foam formation test and foam stability are shown in Table 3. The results indicated that STD-CLG-Dipeptides, STD-CLG-II, and CLG-DI-24h showed no foam formation at a concentration of 5.0 mg/mL. In contrast, CLG-AC-24h and CLG-ACP-24h exhibited foam formation of 2.5 ± 0.01% and 12.5 ± 0.02%, respectively, at the same concentration. Foam formation was observed in all samples at concentrations of 10.0 and 20.0 mg/mL. CLG-AC-24h exhibited a foam formation of 20.00 ± 0.03% at 20.0 mg/mL, which was higher than those of all the other tested samples at the same concentration.

STD-CLG-II and CLG-DI-24h exhibited no measurable foam stability at all tested concentrations. At concentrations of 10 and 20 mg/mL, CLG-AC-24h showed foam stability percentages of 75 ± 0.01% and 100 ± 0.01%, respectively. CLG-ACP-24h exhibited foam stability percentages of 40 ± 0.01%, 83 ± 0.05%, and 100 ± 0.01% at 5.0, 10.0, and 20.0 mg/mL, respectively. These results indicated that only CLG-ACP-24h exhibited measurable foam stability at all tested concentrations. CLG-AC-24h and CLG-ACP-24h showed a significant increase in the percentage of foam stability in a concentration-dependent manner.

**Table 3** Comparison of foaming and foam stability of hydrolysate collagen peptides in each condition

| Samples | Foaming (%) | | | Foam stability (%) | | |
|---|---|---|---|---|---|---|
| | 5.0 mg/mL | 10.0 mg/mL | 20.0 mg/mL | 5.0 mg/mL | 10.0 mg/mL | 20.0 mg/mL |
| STD-CLG-Dipeptides | ND | 2.51 ± 0.01$^{a}$ | 12.50 ± 0.01$^{a}$ | ND | ND | 60 ± 0.01$^{a}$ |
| STD-CLG-II | ND | 7.50 ± 0.01$^{b}$ | 12.51 ± 0.01$^{a}$ | ND | ND | ND |
| CLG-DI-24h | ND | 2.50 ± 0.01$^{a}$ | 12.51 ± 0.01$^{a}$ | ND | ND | ND |
| CLG-AC-24h | 2.5 ± 0.01$^{a}$ | 12.52 ± 0.01$^{c}$ | 20.00 ± 0.03$^{c}$ | ND | 75 ± 0.01$^{a}$ | 100 ± 0.01$^{b}$ |
| CLG-ACP-24h | 12.5 ± 0.02$^{b}$ | 12.51 ± 0.01$^{c}$ | 15.00 ± 0.01$^{b}$ | 40 ± 0.01$^{c}$ | 83 ± 0.05$^{b}$ | 100 ± 0.01$^{b}$ |

**Note:** The data were expressed as mean ± SD (n = 3). ND indicates no foam formation, no measurable foam stability, or values below the detection limit. CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h are the ID names of hydrolysate collagen peptides that were prepared by 6.22 g of the dried powder homogenate bones, incubated in DI water, 0.5 M acetic acid, and 1.0% w/v pepsin, respectively. All conditions were shaken at 120-160 rpm, 37°C for 24 h. The results were analysed using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT). Different superscript letters (a–c) indicate a statistically significant difference ($p < 0.05$).

### 3.7 Antioxidant activity

The results comparing the DPPH radical-scavenging activity of the standard compounds and the hydrolysed collagen peptides are shown in Table 4. All samples were compared at the same final concentration of 25 µg/mL. L-ascorbic acid, used as a standard antioxidant compound, exhibited the highest DPPH radical-scavenging activity among all tested samples (86.41 ± 0.36%). STD-CLG-Dipeptides, STD-CLG-Tripeptides, STD-CLG-II, CLG-AC-24h and CLG-ACP-24h exhibited DPPH radical-scavenging activities of 33.50 ± 1.16, 28.00 ± 0.94, 44.44 ± 5.26, 2.95 ± 7.68, and 31.03 ± 5.45%, respectively, which were significantly lower than that of L-ascorbic acid ($p < 0.05$). Notably, CLG-AC-24h and CLG-ACP-24h showed DPPH radical-scavenging activities comparable to those of STD-CLG-Dipeptides and STD-CLG-Tripeptides, with no significant differences ($p \geq 0.05$). CLG-AC-48h and CLG-ACP-48h exhibited significantly lower DPPH radical-scavenging activities than those of L-ascorbic acid, STD-CLG-Dipeptides, STD-CLG-Tripeptides, STD-CLG-II, CLG-AC-24h, and CLG-ACP-24h ($p < 0.05$). However, CLG-ACL-24h and CLG-PP-24h showed no detectable DPPH radical-scavenging activity at the tested concentrations.

**Table 4**. Comparison of DPPH radical-scavenging activity between standard compounds and hydrolysed collagen peptides

| Conditions | Samples | DPPH radical scavenging activity (%) |
|---|---|---|
| Standard compounds | L-ascorbic acid | 86.41± 0.36[d] |
| | STD-CLG-Dipeptides | 33.5 ± 1.16[a] |
| | STD-CLG-Tripeptides | 28 ± 0.94[a] |
| | STD-CLG-II | 44.44 ± 5.26[b] |
| Control | CLG-DI-24h | 19.54 ± 3.52[c] |
| Acidic (Chemical) | CLG-AC-24h | 32.95 ± 7.68[a] |
| | CLG-AC-48h | 0.91 ± 0.02[f] |
| Enzymatic | CLG-ACL-24h | ND |
| (Biological) | CLG-PP-24h | ND |
| Enzymatic with acidic | CLG-ACP-24h | 31.03 ± 5.45[a] |
| (Chemical + Biological) | CLG-ACP-48h | 2.82 ± 0.03[e] |

**Note:** ND indicates antioxidant values below the detection limit.
STD-CLG-Dipeptides: Collagen dipeptides of fish skin (Japanese collagen dipeptides).
STD-CLG-Tripeptides: Collagen tripeptides of mammalian skin.
STD-CLG-II: Collagen type II of chicken.
CLG-DI-24h: Homogenate bones were incubated in DI water, shaken at 120-160 rpm at 37°C for 24 h
CLG-AC-24h and CLG-AC-48h: Homogenate bones were incubated in 0.5 M acetic acid, shaken at 120-160 rpm at 37°C for 24 and 48 h
CLG-ACL-24h: Homogenate bones were incubated in 1.0% w/v alcalase, shaken at 120-160 rpm at 37°C for 24 h
CLG-PP-24h: Homogenate bones were incubated in 1.0%w/v papain, shaken at 120-160 rpm at 37°C for 24 h
CLG-ACP-24h and CLG-ACP-48h: Homogenate bones were incubated in 1.0% w/v pepsin in 0.5 M acetic acid, shaken at 120-160 rpm at 37°C for 24 and 48 h.
Different superscript letters (a–f) indicate significant differences among treatment groups ($p < 0.05$).

## 4. Discussion

Fish processing by-products represent a sustainable and valuable source of bioactive compounds, particularly collagen and its hydrolysates, which have applications in food, pharmaceutical, and biomedical industries (Kaya et al., 2025).

The present study demonstrates that the extraction method significantly influences the yield, composition, and functional properties of collagen hydrolysate peptides derived from *H. wyckioides* bones. Among the tested conditions, the combined chemical–biological method (CLG-ACP-24h) yielded the highest recovery, suggesting a synergistic effect between acid pre-treatment and enzymatic hydrolysis. The total protein content of CLG-AC-24h and CLG-ACP-24h was approximately 32.24 ± 0.19 and 33.77 ± 0.03%, respectively, which were significantly lower than that of CLG-ACL-24h and CLG-PP-24h ($p < 0.05$). One possible explanation for this decrease in total protein content is the dialysis process. In general, the principle of dialysis is to remove small molecules, such as residual salts or acids used during sample preparation, that are smaller than the pore size of the membrane. In this study, CLG-AC-24h and CLG-ACP-24h were subjected to dialysis using a membrane with a molecular weight cut-off (MWCO) of 3.5 kDa. It is possible that low-molecular-weight hydrolysate collagen peptides were also removed during this process. Therefore, the decrease in total protein content may be attributed to the dialysis process described above. One piece of evidence supporting this hypothesis is that CLG-ACL-24h and CLG-PP-24h, which did not undergo dialysis, exhibited a significantly higher percentage of total protein content than those of CLG-AC-24h and CLG-ACP-24h ($p < 0.05$). Similar trends have been reported in other fish collagen studies,

although the magnitude of yield loss may vary depending on enzyme specificity and membrane cut-off thresholds (Jafari et al., 2020; Gaikwad et al., 2024; Kaya et al., 2025). A possible solution is to modify the pore size of the dialysis membrane to achieve a lower molecular weight cut-off (MWCO), which may help reduce yield loss and increase the percentage of total protein content.

FTIR is a technique commonly used in research to analyse the functional groups present in the structure of interested substances, including collagens. According to Li et al., (2004), an unassociated N–H stretching vibration typically appears between 3400 and 3440 $cm^{-1}$. However, when the N–H group of a peptide participates in hydrogen bonding, this band shifts to a lower wavenumber, generally around 3300 $cm^{-1}$. In this study, the FTIR spectra of standard collagen type II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h showed a band in the range of 3397–3457 $cm^{-1}$, corresponding to the position of an unassociated (free) N–H stretching vibration. This result differs from that reported by Duan et al., (2009) for acid-soluble collagen (ASC) prepared from carp (*Cyprinus carpio*) bone, in which the FTIR spectrum showed a band at 3313.85 $cm^{-1}$, indicating the presence of hydrogen bonding within the collagen structure. In addition, STD-CLG-II and CLG-DI-24h exhibited a C–H stretching band at 2924–2933 $cm^{-1}$, which was not observed in CLG-AC-24h and CLG-ACP-24h. Muyonga et al., (2004) indicated that this IR band corresponds to the C–H groups of the collagen backbone. The decrease in intensity, or absence, of the C–H stretching band in CLG-AC-24h and CLG-ACP-24h may be associated with the cleavage of bonds within the main collagen chain, resulting in a conformational change into short peptide chains. This, in turn, may account for the band being undetectable or poorly resolved. Similarly, CLG-DI-24h and STD-CLG-II exhibited a band at 1238 and 1242 $cm^{-1}$, respectively. However, this band was absent in CLG-AC-24h and CLG-ACP-24h. This result is consistent with the report by Kittiphattanabawon et al., (2012), in which high-molecular-weight collagen was shown to exhibit a band at 1230–1245 $cm^{-1}$. The above findings suggest that CLG-AC-24h and CLG-ACP-24h were converted into hydrolysed collagen peptides through the catalytic mechanism of peptide bond hydrolysis by acid and the enzyme pepsin (Govindharaj et al., 2019; Kaya et al., 2025). Furthermore, the amide I band generally occurs within the range of 1625–1690 $cm^{-1}$, while the amide II band typically appears within the normal absorption range of 1550–1600 $cm^{-1}$ (Duan et al., 2009). Based on the FTIR spectra, bands were detected at 1653, 1655, 1646, and 1643 $cm^{-1}$ for STD-CLG-II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h, respectively, indicating the position of the amide I band in these samples. More importantly, an amide II band was also detected in CLG-AC-24h and CLG-ACP-24h, both appearing at a similar position of 1561 $cm^{-1}$ (Duan et al., 2009; Jafari et al., 2020).

Fibrillar collagens, such as types I and II, are composed of repeating Gly-X-Y triplets, in which the X and Y positions are frequently occupied by proline and hydroxyproline, respectively (Berillis, 2015). The presence of hydroxyproline is particularly significant as it is unique to collagen and plays a central role in stabilizing the triple helix through hydrogen bonding (He et al., 2019). Analysis of the amino acid composition of collagen from different sources revealed variations in amino acid proportions. The amino acid composition of acid-soluble collagen from calf skin, cod skin, and carp (including carp scale and bone) showed the highest proportion of glycine among all amino acids present. Proline, alanine, and hydroxyproline were detected in decreasing order of proportion, respectively (Duan et al., 2009). In this study, the amino acid groups in the samples were preliminarily examined using paper chromatography. The $R_f$ values of the amino acid spots in collagen type I, collagen type II, CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h were like those of the standard amino acids glycine, hydroxyproline, proline, alanine, leucine, and isoleucine. This result is consistent with the findings reported by Duan et al., (2009). However,

the amino acid analysis performed in this study was qualitative, serving as preliminary data to guide future quantitative analysis of amino acid composition using more advanced analytical techniques.

The percentage of transmittance of the prepared hydrolysed collagen peptide samples and standard collagen type II (STD-CLG-II) was analysed to assess sample cloudiness in solvents at different pH levels. Generally, collagen is a fibrous protein with a large molecular size and is poorly soluble in water (Nelson & Cox, 2005; Espinales et al., 2023). Consequently, a cloudy appearance is commonly observed when high-molecular-weight collagen is dissolved in water. This effect can pose challenges for product development. The results showed that, in deionized water, CLG-AC-24h and CLG-ACP-24h exhibited percentages of transmittance of 95.97% and 94.80%, respectively, which were higher than those of STD-CLG-II and CLG-DI-24h. A similar trend was observed for the percentage of transmittance of the samples in a solvent with low pH (high acidity). Therefore, acid and enzymatic hydrolysis related to reduced collagen size enhancing the turbidity decrease. This data may support the development of beverages, liquid nutritional, and functional food products in the future.

The foaming capacity and stability of proteins are relevant functional properties for applications in aerated food systems such as ice cream, whipped creams, and meringues (Kittiphattanabawon et al., 2015). The results of this study showed that CLG-AC-24h and CLG-ACP-24h formed foam in a concentration-dependent manner, similar to STD-CLG-Dipeptides and STD-CLG-II. However, the foam formation percentage was relatively low, which might be related to the sample concentration and the molecular size of the protein or peptide (Naghdi et al., 2023). The percentage of foaming stability is related to foam formation and depends on the sample concentration. However, the foam stability of STD-CLG-II was relatively low, which might be due to its solubility behaviour.

UV-Vis spectrophotometric analysis provided additional evidence of the purity of the extracted collagen peptides. The absence of a significant absorbance peak at 260–280 nm in CLG-DI-24h, CLG-AC-24h, and CLG-ACP-24h. The absence of a distinct peak at 260–280 nm suggests a relatively low abundance of aromatic residues or other UV-absorbing components; however, this observation alone does not confirm sample purity.

The DPPH radical scavenging assay revealed that hydrolysate collagen peptides from *H. wyckioides* bones possess moderate antioxidant activity. CLG-AC-24h and CLG-ACP-24h exhibited antioxidant efficacy comparable to standard collagen dipeptides and tripeptides, which are known bioactive fractions. The antioxidant activity of collagen peptides is primarily attributed to specific amino acid sequences—particularly those rich in proline, glycine, and hydroxyproline—which can donate hydrogen atoms to neutralize free radicals (Ngo et al., 2010). However, CLG-ACL-24h and CLG-PP-24h lack antioxidant measurable activity at 500 μg/mL. The antioxidant inactivity of CLG-ACL-24h and CLG-PP-24h might be related to their amino acid sequence and size. Using just one type of protease may result in hydrolysed collagen peptides with a high molecular weight. Thus, only papain or alcalase may still not be suitable for preparing hydrolysate collagen peptide from *H. wyckioides* bone. The large molecular size of the peptides may affect the effectiveness of DPPH radical scavenging. This finding is consistent with the report by Ishak and Sarbon (2018), who demonstrated that hydrolysed peptides with a lower molecular weight exhibited greater antioxidant activity.

Collectively, the findings of this study demonstrate that *H. wyckioides* bone waste is a viable and underutilized source of hydrolysate collagen peptides. The chemical and combined chemical-biological method offers the most favorable balance of yield, foaming stability, and antioxidant activity. The dialysis process can remove salt and small molecules. Thus, this step may decrease the geosmin and 2-MIB, which is critical for consumer acceptance of freshwater fish-derived collagen products. Future research should

focus on: (1) optimization of biological extraction conditions (enzyme concentration, pH, temperature) for improved color and texture; (2) fractionation of active peptides; (3) evaluation bioavailability and safety of hydrolysate collagen peptide in vitro and in vivo and (4) scale-up studies to assess industrial feasibility. The valorization of fish processing by-products, such as bones of *H. wyckioides*, aligns with circular economy principles and contributes to sustainable aquaculture value chains. These data support the classification of the isolated peptides as collagen-derived, reinforcing the suitability of *H. wyckioides* bone waste as a source for collagen peptide production.

## 5. Conclusions and recommendations

*H. wyckioides* bones are a collagen source that can be extracted using various conditions (chemical, biological, and combined methods). Among the conditions tested, CLG-ACP-24h produced the highest collagen peptide yield, while CLG-ACL-24h showed the highest total protein content. The FTIR spectrum of the prepared samples showed functional groups like those detected in the standard collagen. CLG-AC-24h and CLG-ACP-24h exhibited foaming properties, and foam stability was concentration-dependent. The percentage of transmittance in water and trifluoroacetic acid was higher than that of collagen type II. The amino acids identified in the sample separated by paper chromatography — glycine, proline, and hydroxyproline, exhibited $R_f$ values close to those of the standard amino acids. Solutions of both samples (CLG-AC-24h and CLG-ACP-24h) exhibited maximum UV absorption at 200–220 nm, similar to that observed for the standard collagen type II solution; however, both samples showed a low UV absorption signal at 280 nm. CLG-AC-24h and CLG-ACP-24h exhibited DPPH radical-scavenging activity that was not significantly different from that of the standard collagen dipeptide solution (CLG-STP-Dipeptides). These findings offer preliminary support for the potential application of fish bone from *H. wyckioides* in the development of collagen-based products.

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## 7. References

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