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Ultrasonic-Assisted Enzyme Hydrolysis of Chicken By-Products: Optimization, Peptide Mapping and Antioxidative Efficacy by In-vitro and In-silico Approach

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ABSTRACT

This study investigates the ultrasound-assisted enzymatic extraction of collagen hydrolysates (CH) from chicken skin, heads, and feet, aiming to generate bioactive peptides with potent antioxidant activity. Process optimization involved acetic acid pre-treatment, ultrasonication (39 kHz), and enzymatic hydrolysis using collagenase, followed by ultrafiltration (<10 kDa). The optimized treatment (T2) yielded hydrolysates with a high degree of hydrolysis (85.31%) and protein content (11.73 g/dL). SDS-PAGE revealed predominantly low-molecular-weight peptides, indicative of effective collagen breakdown. Antioxidant capacity, measured via DPPH and ABTS+ assays, was significantly elevated in T2 (76.38% and 91.41%, respectively). Peptidomic profiling was conducted using LC-ESI-MS/MS on a QTOF mass spectrometer, enabling the identification of numerous peptide sequences. In-silico bioactivity prediction using the BIOPEP database and Peptide Ranker algorithm revealed multiple sequences with high antioxidant potential, particularly short-chain peptides rich in hydrophobic and aromatic residues. The integration of advanced proteomic tools and bioinformatic analyses confirmed the efficacy of ultrasound-assisted enzymatic hydrolysis in producing functionally relevant peptides. These findings support the valorization of poultry by-products as a sustainable source of antioxidative peptides for potential applications in food and health sectors.

Keywords: Collagen hydrolysate; SDS-page; Mass spectrometer; In-silico approach; Peptide ranking

INTRODUCTION

The poultry industry produces vast quantities of by-products such as chicken skin, heads, and feet, which are often discarded or used in low-value applications despite being rich in structurally important proteins like collagen. Collagen, a fibrous protein consisting predominantly of glycine, proline, and hydroxyproline, serves as a precursor to bioactive peptides that exhibit diverse physiological functions such as antioxidant, antihypertensive, and antimicrobial activities (Barzideh et al. 2014; Zhuang et al. 2009). Recent efforts have focused on converting such underutilized by-products into value-added functional ingredients, aligning with the principles of a circular bioeconomy (Banerjee et al. 2024). Collagen hydrolysates (CHs) are typically obtained through chemical or enzymatic hydrolysis. Among these, enzymatic hydrolysis is preferred for its mild processing conditions and ability to yield peptides with high bioactivity. However, the rigid triple-helical structure of collagen often limits enzymatic efficiency. To overcome this, ultrasound-assisted enzymatic hydrolysis (UAEH) has emerged as a promising green technology that enhances protein solubility, disrupts collagen fibrils, and increases enzyme accessibility (Akram et al. 2020; Zhang et al. 2020). UAEH has been shown to improve the degree of hydrolysis and enhance the

antioxidant properties of the resulting peptides (Hernández-Morales et al. 2023; Wei et al. 2022). Analytical techniques play a pivotal role in characterizing collagen hydrolysates. SDS-PAGE and FTIR spectroscopy help evaluate molecular fragmentation and conformational changes, respectively (Akram et al. 2020). However, for precise peptide identification, liquid chromatography coupled with tandem mass spectrometry (LC-ESI-QTOF-MS/MS) is considered the gold standard. It enables accurate sequencing and detection of low-molecular-weight peptides, many of which are associated with health-promoting bioactivities (Xie et al. 2020; Zhao et al. 2019).

Given the diversity of peptides generated from collagen, in-vitro validation of all sequences is resource-intensive. In this context, in-silico tools such as BIOPEP-UWM and Peptide Ranker offer predictive insights into the potential bioactivity of identified sequences. These tools assess sequence motifs and physicochemical properties to estimate antioxidant, ACE-inhibitory, or antimicrobial potential (Mooney et al. 2012; Minkiewicz et al. 2008). When integrated with mass spectrometric data, these platforms facilitate the prioritization of peptides for further functional validation. While marine and bovine collagen sources are extensively studied, poultry by-products offer an economical and sustainable alternative. Chicken feet, heads, and skin are particularly rich in type I and II collagen and have shown comparable functionality to mammalian sources in terms of antioxidant and emulsifying properties (Lin and Liu, 2006; Banerjee et al. 2024). Furthermore, the blending of ultrasound and enzymatic hydrolysis has proven effective in increasing extraction efficiency, peptide yield, and functional performance of hydrolysates (Wei et al. 2022). In this study, collagen was extracted from chicken skin, heads, and feet using a combination of acid pretreatment, ultrasound-assisted enzymatic hydrolysis, and ultrafiltration. The resulting hydrolysates were evaluated for their antioxidant potential using DPPH and ABTS assays, and peptide profiles were analyzed using LC-ESI-QTOF-MS/MS. Bioinformatic tools were employed to predict the antioxidant potential of the sequenced peptides. This work contributes to the sustainable valorization of poultry processing waste and the identification of novel antioxidant peptides with possible applications in functional foods or nutraceutical formulations. (Figure 1)

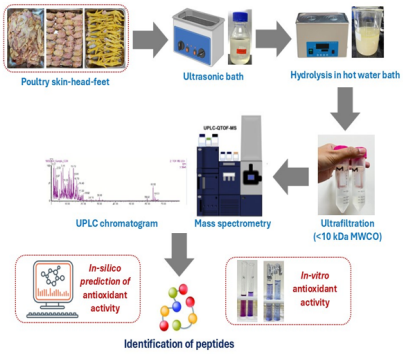

Figure 1. Graphical representation of the research work

MATERIALS AND METHODS

The purpose of this study is to extract collagen hydrolysates from poultry waste and to determine their bioactivities by in-vitro and in-silico approach. The research was conducted at the Meat Proteomics Laboratory, Indian Council of Agricultural Research-National Meat Research Institute, Hyderabad (ICAR-NMRI), April to December, 2024.

Collection and preparation of raw materials: 50-day-old broiler chicken were slaughtered in accordance with routine procedures at the Poultry Processing Plant of ICAR-National Meat Research Institute (ICAR-NMRI), and the skins, head, and feet were collected. Following a one-minute scalding at 55 °C, all of the feathers were manually removed from the skin samples. All the separable fat, fascia, and attached meat remnants were scraped off the skins and cleaned with potable tap water. About 500 g of washed and cleaned skins were placed in low-density polyethylene (LDPE) packs and frozen at -18°C until further studies. Before being used, frozen and thawed chicken skin was chopped into pieces and cleansed with tap water. Chicken heads were collected and washed with potable water to clean all the dirt, debris, and feathers. After washing, chicken heads were allowed to dry, processed by discarding the beaks and combs and cutting them into pieces followed by mincing in a meat mincer. The minced sample was kept in LDPE packs and frozen at -18°C. Chicken feet were collected and washed with running tap water, allowed to dry, denailed and cut into small pieces. Finally, the samples were minced with a meat mincer and kept in LDPE packs at -18°C.

Ultrasound-assisted enzymatic extraction: In order to prepare the samples for ultrasound-assisted extraction using an ultrasonic water-bath, chicken skin-head-feet (SHF) samples were treated with 3% acetic acid in a 1:1 (w/v) ratio for nine hours. The samples were then rinsed and filtered with muslin cloth until their pH reached 7.5 to 8. The acid-pretreated chicken SHF sample was placed in 500 ml glass bottles, and distilled water was added to the

samples (1:2 w/v) for ultrasound pre-treatment using an ultrasonic water bath (Sonica Ultrasonic Cleaner 2200 S3, SOLTEC ®, Italy). The samples were then placed in the water bath's stainless steel beaker holder. For one h, the ultrasonic water bath was run at 250 W with an ultrasound frequency of 39 kHz ± 1 kHz. The enzyme Collagenase (1:1000 w/w) was added to the sample and the hydrolysis was carried out in a water bath at 40 °C for three hours. The enzyme was then inactivated heating the sample to 90 °C for 10 min. Collagen hydrolysates (CH) were collected, filtered, and stored at 4 ± 1 °C until further analysis.

Lyophilization and ultrafiltration of hydrolysates: The hydrolysates were divided into two fractions. Of these, one fraction was lyophilized in a laboratory-scale freeze dryer/lyophilizer (IGLZ20, IGENE LABSERVE, Ahmedabad, India) and stored at 4 °C. Prior to lyophilization, the sample was first frozen at -80 °C for 24 hrs. Then, the samples were freeze-dried under 0.001 Pa vacuum at -58 °C (heating shelf temperature). The dried hydrolysate obtained was referred to as freeze-dried/lyophilized collagen hydrolysate. The other fraction was ultracentrifuged with Amicon® Ultra Centrifugal Filter, 10 kDa MWCO at 6,000 rpm for 30 min at 4 °C.

Degree of hydrolysis (DH%): The degree of hydrolysis of hydrolysates was assessed by following the method described by Maheswarappa et al. (2022), with some modifications. Briefly, 2 ml aliquot of the aqueous suspension of hydrolysates were mixed with 2 ml of 10 % TCA followed by centrifugation at 15,770 rpm for 15 min at 4 °C. The protein concentration of the hydrolysates, as well as the supernatant from respective CH samples, was assayed by using the Biuret method by adding 1 ml of hydrolysate/supernatant with 5 ml of Biuret reagent in the test tubes and kept in the dark for 30 min at room temperature. The percentage DH was expressed

DH % = PC of hydrolysate−PC of supernatant / PC of hydrolysate

Where, PC = Protein concentration

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): The SDS-PAGE was carried out using the method of (Laemmli 1970) with mini-gel electrophoresis apparatus (Mini-PROTEAN® 3, BioRad Laboratories, Hercules, CA, USA) for all the collagen hydrolysate samples. The sample was prepared by mixing 10 µL, 12µL and 15µL of collagen hydrolysate from each sample with 10 µL of 1x Laemmle buffer and was loaded onto 12 % acrylamide gel after denaturation by boiling at 100 °C for about 5 min. Electrophoresis was performed at a constant voltage mode of 80 V/slab at 60 mA for 2 h or till the dye front reached 0.5 cm from the lower edge of the gel. The gel was stained with Coomassie brilliant blue staining solution (0.2 % Coomassie brilliant blue, 40 % methanol and 10 % acetic acid) for 1 h followed by destaining for 45 minutes by using distaining solution (40 % methanol and 10 % acetic acid). The CH samples from all the groups were run together under the same conditions.

Profiling of hydrolysates by mass spectrometry: Electrospray ionization (ESI) liquid chromatography-mass spectrometry (LC-MS) method was used for profiling of the ultrafiltered collagen hydrolysate. Acquity Ultra Performance Liquid Chromatography (UPLC) connected to Waters Xevo G2 XS QTOF Mass Spectrometry, (USA) was used for the mass spectrometry analysis. About 100µl of the sample was reduced with 100 mM DTT at 95°C for 1h at 400rpm and alkylated with 250 mM IDA with incubation in dark at room temperature for 45 min. The sample was incubated with Trypsin at 37°C overnight. The resulting samples were vacuum-dried and dissolved in 50µl of 0.1% formic acid in water. Desalting of the peptides was carried out. After centrifugation at 13000g the supernatant of 10µl sample was injected into C18 UPLC column for separation of peptides followed by analysis on the Q-TOF instrument for MS and MSMS. The raw data was processed by MassLynx 4.1 WATERS. The individual peptides of MSMS spectra were matched to the theoretical tryptic peptides.

In-vitro antioxidant activities of collagen hydrolysate DPPH radical scavenging activity: The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity was determined using the method of Brand-Williams et al. (1995), with some modifications. One ml of 100 µM DPPH (dissolved in ethanol), 0.25 ml of 0.1 M Tris- HCl buffer (pH-7.5), and 25 µL of sample solution were mixed properly in test tubes and the absorbance was taken at 0 min at 517 nm using UV

spectrophotometer (Model: UV-1700 Pharma-Spec, SHIMANZU, Japan). The above samples were also kept at room temperature under dark conditions for measurement of second absorbance after 20 min and ethanol was taken as a blank. The percent inhibition of DPPH radical scavenging activity was determined using the equation.

% inhibition of DDPH radical scavenging activity= $100 - (At_{20} / At_0) \times 100$

Where, At_0 = Absorbance at 0 min At_{20} = Absorbance at 20 min

$ABTS^+$ radical scavenging activity: The $ABTS^+$ (2,2-Azino-bis,3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt) radical scavenging activity was determined according to Salami et al. (2010). Fresh solution of the $ABTS^+$ was prepared in distilled water to a concentration of 7 mM. Freshly prepared $ABTS^+$ stock solution was mixed with the same quantity of 2.45 mM potassium persulphate (K2S2O8) solution and the mixture was kept in the dark

room for 16 h before use for the formation of $ABTS^+$ radical cation ($ABTS^+$). Before use, the prepared mix solution absorbance was adjusted at 0.7 with 5mM sodium phosphate buffer (pH-7.4). One ml of ABTS+ working solution (which was adjusted to 0.70) was well mixed with 30 µL of sample solution and kept in the dark at room temperature for 20 min and the absorbance was taken at 732 nm using UV spectrophotometer (Model: UV-1700 Pharma-Spec, SHIMANZU, Japan). Sodium phosphate buffer (pH-7.4) was used as a blank. The $ABTS^+$ activity was then calculated using the formula:

% inhibition of $ABTS^+$ activity $=0.7-At_{20}$ /0.7 X100

Prediction of bioactivity through in-silico approach: The prediction of peptide profile identified from LC-MS/MS analysis was carried out using BIOPEP database (available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (Minkiewicz et al. 2008) using 'profiles of potential biological activity' tool. The probability of peptides with bioactivity was predicted by the Peptide Ranker tool, which was accessed a http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php (Mooney et al. 2012). All peptides with scores >0.5were considered to be bioactive.

Statistical analysis: All experiments were performed in triplicate, and the results were reported as mean ± SD. The data were analyzed by the one-way ANOVA test using statistical analysis software (SPSS version 26.0 for windows; SPSS, Chicago, IL, U.S.A.). The results were considered statistically significant for P values less than 0.05.

RESULTS AND DISCUSSIONS

The studies have been done for preparation of poultry waste-based collagen hydrolysates. The present section describes the outcome of the experiments conducted during the study.

Preparation of collagen hydrolysate from poultry waste by enzymatic hydrolysis: In the present study, the above-mentioned sample i.e. chicken skin-head-feet (SHF) blend was chosen for ultrasound assisted - enzymatic hydrolysis to obtain collagen hydrolysates (CH). For optimization of the process, initially, the samples were subjected to 9 h of 3% acetic acid (1:100) pre-treatment followed by 1 h of ultrasonic bath-assisted extraction for better fragmentation of collagen tissues. Collagenase enzyme (1:1000) was added to each sample and the water bath hydrolysis was carried out for 2, 3 and 4 h to get the optimum condition for hydrolysis. After hydrolysis the collagen hydrolysates (CH) were filtered and the degree of hydrolysis and total protein estimation were done to select the treatment for further analysis. Table.3.1. displayed the total protein and the degree of hydrolysis of the sample (2, 3, and 4 h water bath hydrolysis)

Table 1. Optimization of the time (hrs) for collagen hydrolysate preparation

Hydrolysates	Total protein (g/dL) (TP)	Degree of hydrolysis (%) (DH)
2h	3.98 ± 0.11^b	40.00 ± 3.60^a
3h	5.91 ± 0.02^c	51.33 ± 1.52^b
4h	3.71 ± 0.04^a	48.66 ± 1.52^b

**Mean values (±SD) with different superscripts treatment-wise differed significantly (p< 0.05)*

Based on the results of the degree of hydrolysis and total protein estimation, the 3 hours enzyme-sonication treated CH showed significantly (P<0.05) higher degree of hydrolysis with (51.33%) and total protein (5.91g/dL) so, was selected and named as T1 for further analysis. As the 3h (T1) of treatment was selected for production of collagen hydrolysates (CH), so the optimized collagen hydrolysate (T1) was ultrafiltered with Amicon® Ultracentrifugal filter, 10 kDa MWCO at 6000 rpm for 30 min at 4 °C, and the filtrate was denoted as T2 and another treatment was kept as control (C) where there was no use of ultrasonication or ultrafiltration, it was just simple water bath hydrolysis for 3 h without enzyme.

Degree of hydrolysis and total protein estimation: The degree to which a protein has been hydrolyzed reflects the number of peptide bonds broken and, therefore, the average size of the peptides present. The number of peptide bonds cleaved as a proportion of the total number of peptide bonds present is the degree of hydrolysis (DH) (Rutherfurd, 2010).

Table 2. Degree of hydrolysis and total protein estimation of collagen hydrolysates

Hydrolysate	Total protein(g/dL) (TP)	Degree of hydrolysis (%) (DH)
C	3.51 ± 0.30^b	31.33 ± 1.23^c
T_1	4.20 ± 0.23^b	54.83 ± 2.95^b
T_2	11.73 ± 0.52^a	85.31 ± 2.01^a

**Mean values (±SD) with different superscripts treatment-wise (small letters) differed significantly (p< 0.05)*

C = Control (No Enzyme + No ultrasonication) collagen hydrolysate; T1= Enzyme + Ultrasonication Treated collagen hydrolysate and T2 = Enzyme + Ultrasonication + Ultrafiltrate collagen hydrolysate obtained from T1

The total protein concentration (TP) and degree of hydrolysis (DH%) of T1 and T2 were compared to Control

(C) sample, i.e. chicken SHF blend subjected to 3 h hydrolysis without any ultrasound or enzyme treatment. The results (Table.3.2; Fig.4.3) revealed that the T2 is having significantly ($P < 0.05$) higher DH (85.31%) and TP (11.73 g/dL) when compared with C (DH = 31.33%; TP = 3.51 g/dL) and T1 (DH = 54.83%; TP = 4.20 g/dL). This increase can be attributed to the synergistic effect of ultrasound pre-treatment, enzymatic hydrolysis, and ultrafiltration, which collectively enhanced collagen breakdown. Ultrasound-assisted enzymatic hydrolysis (UAEH) has been shown to improve the structural accessibility of enzymes by disrupting the dense, triple-helical collagen network via acoustic cavitation, thus enhancing enzymatic activity and increasing DH (Zhang et al. 2020). Similar improvements were reported by Wei et al. (2022), where ultrasound pre-treatment led to significantly increased DH in chicken skin collagen hydrolysates. The combination of mechanical disruption and enzymatic specificity resulted in the generation of shorter peptides and higher protein solubilization, reflected by the higher protein concentration in T2 (11.73 g/dL) observed in this study. The application of ultrafiltration in T2 further contributed to the enrichment of low-molecular-weight peptides (<10 kDa), which are known to possess higher functional properties, particularly antioxidant activity, due to their improved bioavailability and capacity to interact with free radicals (Sila and Bougatef, 2016). Such peptides have been widely reported to exhibit enhanced solubility and absorption, making them highly desirable in nutraceutical and functional food applications (Udenigwe and Aluko, 2012). The higher DH observed in T2 is in agreement with previous findings that indicated increased hydrolysis rates when enzymatic treatments were coupled with physical pretreatments like ultrasonication (Akram et al., 2020).

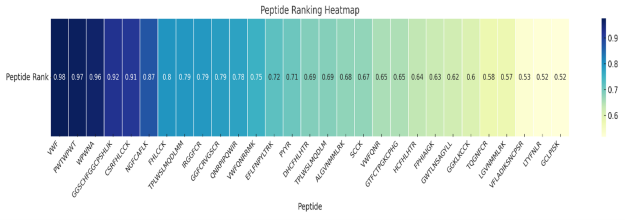

Figure 4. Heat map presenting the ranking of the peptides according to their bioactivity

SDS-PAGE profile of collagen hydrolysate obtained by different treatments: The molecular weight distribution of control, T1, and T2 collagen hydrolysates was demonstrated in Fig.3.2. The SDS-PAGE results reveal distinct peptide profiles among the three treatments—Control (C), enzyme + ultrasound treated (T1), and ultrafiltrate of T1 (T2). The gel patterns demonstrate a visible shift toward lower molecular weight bands in T1 and T2 compared to the control, indicating effective degradation of high-molecular-weight collagen into smaller peptides.

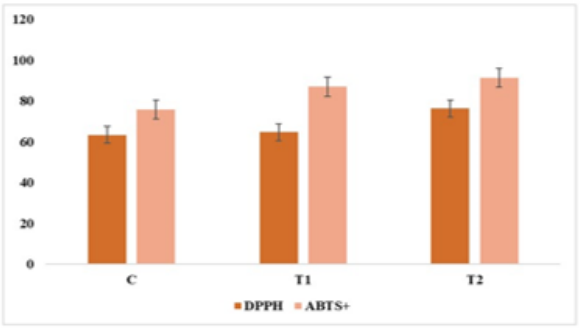

Figure 3. DDPH & ABTS+ of the collagen hydrolysates

In the control lanes, intense bands are observed at higher molecular weights (>48 kDa), suggesting the presence of intact or partially hydrolyzed collagen chains. This aligns with the absence of enzymatic and ultrasound treatment, which are essential for disrupting the triple-helical collagen structure (Zhang et al. 2020). In contrast, the T1 samples (enzyme + ultrasound) display a broader range of peptide fragments, including prominent bands below 25 kDa. This fragmentation reflects successful hydrolysis promoted by ultrasound-induced cavitation, which improves enzyme accessibility and enhances cleavage efficiency (Akram et al. 2020; Udenigwe and Aluko, 2012). Most notably, the T2 lanes (ultrafiltrated T1 hydrolysate, <10 kDa) exhibit faint or no bands above 20 kDa, and instead are enriched in peptides below 17 kDa. The reduced intensity and molecular size distribution confirm the removal of larger peptides and the concentration of bioactive, low-molecular-weight peptides. Sila and Bougatef (2016) emphasized that low molecular weight peptides (<10 kDa) often show enhanced bioactivities, including antioxidant, antimicrobial, and ACE-inhibitory properties, due to their improved solubility, faster absorption, and better radical-scavenging efficiency. Furthermore, this distribution shift corresponds with the observed increase in degree of hydrolysis (DH%) in T1 and T2. A higher DH generally reflects a higher proportion of cleaved peptide bonds, leading to shorter peptide chains (Rutherfurd 2010). Zhao et al. (2018) also demonstrated that extensive enzymatic hydrolysis of chicken collagen generated peptide bands primarily under 20 kDa, which were functionally more potent than their high-molecular-weight counterparts. Taken together, the SDS-PAGE profile confirms that ultrasound-assisted enzymatic hydrolysis, especially when followed by ultrafiltration, is highly effective in producing low-molecular-weight peptides from poultry collagen. These fractions are not only biochemically distinct but also potentially enriched in bio functional activity. (Figure 2)

Determination of bioactivities of collagen hydrolysates and its fraction: The antioxidative activities of CH prepared from chicken SHF blend prepared using different methods was determined by different assays. ABTS+ and DPPH radical scavenging assays are based on a single electron transfer mechanism followed by neutralization via electron transfer and/or quenching (hydrogen atom transfer/HAT) (Esfandi et al. 2019). The ABTS+ assay is a colorimetric assay that evaluates the potential of an antioxidant to inhibit the formation of a colored radical cation ABTS+, a blue-green chromophore with the characteristic absorption at 734 nm. The ABTS+ is generated by the oxidation of ABTS+ with potassium persulphate and reduced in the presence of hydrogen-donating antioxidants or chain-breaking antioxidant When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability, thereby terminating the radical chain reaction (Raghavan et al. 2008).

The T2 has significantly ($P \leq 0.05$) higher DPPH % (76.38%) and ABTS % (91.41%) values compared to Control and T1 (Fig.3.3). The biological functions of peptides are significantly influenced by their amino acid composition, sequence, molecular size, and spatial conformation, which are considered crucial for their biological activities (Ma et al. 2018; Agrawal et al.2018). Antioxidant peptides function as lipid peroxyl radical scavengers and hydrogen donors (Zhao et al. 2018). Short peptides, typically comprising 2 to 10 amino acids, often exhibit higher activity compared to their native proteins due to their ability to readily interact with reactive radicals, thereby enhancing their functionality within reaction systems (Feng et al. 2018). Variations in the antioxidant activities of the hydrolysates observed in this study may be attributed to differences in amino acid composition and chain length of peptides (Chi et al. 2014; Li et al. 2013). The ultrafiltrate fraction (<10 kDa) of the hydrolysate, such as T2, containing smaller molecular weight peptides, is likely more effective at accessing and neutralizing free radicals (Sila and Bougatef, 2016). (Figure 3)

Identification of bioactive peptides by mass spectrometry from hydrolysate fractions: The antioxidant activities of T_2 were found significantly higher than the C and T_1 and subjected to mass spectrometry for the identification of bioactive peptides. Table.3.3 listed the peptide detected in the T_2 treatment showing antioxidant activities.

The prediction of bioactive peptide profile identified from LC–ESI/MS/MS analysis was carried out using BIOPEP database (available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (Minkiewicz et al. 2008). The peptide profiling of ultrafiltered collagen hydrolysates (T2) using LC–ESI–QTOF–MS/MS revealed a diverse spectrum of short-chain peptides derived from the ultrasound-assisted enzymatic breakdown of chicken skin-head-feet collagen. High-resolution QTOF-MS enabled accurate detection of multiply charged ions, and subsequent MS/MS fragmentation confirmed the presence of low-molecular-weight peptides, predominantly under 10< kDa. This observation is consistent with the SDS-PAGE profile and high degree of hydrolysis (85.31%) obtained for T2. Several prominent peptides identified through spectral matching included VWFQNR, PWTWPWT, WPWNA, and GTFCTPGK. These sequences were characterized by the presence of hydrophobic and aromatic amino acids, such as valine (V), phenylalanine (F), tryptophan (W), and proline (P), which are widely recognized for enhancing the bioactive properties of peptides, particularly antioxidant capacity (Udenigwe and Aluko, 2012).

To evaluate potential biological activity, the identified peptides were analyzed using the Peptide Ranker tool. Many peptides yielded scores above 0.7, indicating high probability of bioactivity. For instance, VWF (0.976), PWTWPWT (0.970), and WPWNA (0.959) demonstrated strong predictive scores. These peptides fall within the typical range of bioactive sequences (2–10 amino acids) and exhibit features, such as hydrophobicity and low molecular weight, that favor free radical scavenging, metal ion chelation, and rapid gastrointestinal absorption (Dai et al. 2021; Sila and Bougatef, 2016).

The production of such peptides is directly influenced by the application of ultrasound in the hydrolysis process. Ultrasonication facilitates cavitation and microstreaming, which disrupt the collagen's triple-helical structure and enhance enzymatic access, resulting in more extensive cleavage (Zhang et al. 2020). Furthermore, the use of ultrafiltration helped to selectively concentrate the low-molecular-weight peptide fraction, improving both the purity and functionality of the final hydrolysate.

Collectively, the MS-based profiling confirmed the generation of multiple bioactive peptides in the T2 hydrolysate, validating the effectiveness of the ultrasound–enzyme–ultrafiltration approach. These findings align with previous studies that demonstrate how targeted enzymatic hydrolysis of animal by-products can yield peptides with promising nutraceutical applications (Akram et al. 2020; Barzideh et al. 2014).

The amino acid profile of small peptides and the position of amino acids in the peptide sequence are responsible for bioactivity. Hence, the BIOPEP tool was also used to calculate the location of the sequence of peptides majorly

responsible for antioxidant activities. The combination of *in-vitro* and *in-silico* methods can increase the identification of bioactive peptides for further applications in food and human health. Hence, the cost-effective and time-saving *in-silico* method was also used to predict the bioactivity of the theoretically released peptides derived from ultrasound-assisted extraction of collagen hydrolysates from chicken Skin-Head-Feet blend.

Table 3. List of antioxidative peptides in chicken SHF hydrolysates (<10kDa ultrafiltrate)

Peptide seq	Number of peptides	Sequence	Location
VWFQNRRMK	1	VW	1-2
ERERW	1	RW	4-5
VWFQN	1	VW	1-2
VWFQNR	1	VW	1-2
VWF	1	VW	1-2
PWTWPWT	5	TW, PT, PT, PWT, PW	3-4, 1-3, 5-7, 1-2, 5-6
TQGNFCR	1	FC	5-6
VDEDLQGAGGIQ	1	GA	9-10
EGLPMTGAVQEMR	2	LPM, GA	3-5, 7-8
LGCCVVEKPQPKK	2	LPM, GA	3-5, 7-8
TPLWSLMQDLMM	3	LWS, LW, MM	3-5, 3-4, 11-12
TPLWSLMQDLM	1	LW	3-4
GWTLNSAGYLL	4	YL, YLL, GW, YL	9-10, 9-11, 1-2, 9-10
PYYR	2	PYY, YYR	1-3, 2-4
TQGSHNK	1	GSH	3-5
GTFCTPGKCPHG	2	FC, PH	3-4, 10-11
MELEAMSRYTS	2	EL, RY	2-3, 8-9
MELEAMSR	1	EL	2-3
VAALPGAR	1	GA	6-7
VFLADIKSNCPSR	1	VFL	1-3
FYNDLQQYLNVVTRHR	2	RHR, FY	14-16, 1-2
VLCEEPMGAA	2	GAA, GA	8-10, 8-9
GGSCHFGGCPSHLIK	2	HL, GC	12-13, 8-9
WPWNA	1	PW	2-3
GGKLKCCK	1	LK	4-5
TSPWLLAPGNNPH	3	PWL, PW, PH	3-5, 3-4, 12-13
GGFCRVGSCR	1	FC	3-4
FPHIAIGK	2	PH, PHI	2-4, 2-3
IRGGFCR	2	IR, FC	1-2, 5-6
CSRFHLCCK	1	HL	5-6
NGFCAFLK	2	LK, FC	7-8, 3-4
FHLCCK	1	HL	2-3
CKNSNTVYCK	1	VY	7-8
NTVYCKLESCPSR	1	VY	3-4
GQGKPS	1	KP	4-5
ALGVNMMLRK	1	MM	6-7
LGVNMMLRK	1	MM	5-6
ILNLTERQ	1	LT	4-5
QNRPIPQWIR	1	IR	11-12
DHCFHLHTR	4	LH, HL, LHT, HC	6-7 ,5-6, 6-8,2-3
HCFHLHTR	4	LH, HL, LHT, HC	5-6, 4-5,5-7,1-2
TVYD	1	VY	2-3
EFLFNPYLTRK	2	YL, LT	7-8, 8-9
GRQTYSR	1	TY	4-5
GCLPISKEVNR	1	GC	1-2
MIYQPRQTIWV	1	IY	2-3
GCLPISK	1	GC	1-2
MQILTLLFAVLLLMLR	1	LT	4-5
GLPQDCERRGGFCSHK	1	FC	12-13
RGGFCSHK	1	FC	4-5
TLTSDPR	1	LT	2-3
MSKVTFKVTLTSDPR	1	LT	10-11
YLFAYAGIKY	3	AY, YA, YL	4-5, 5-6,1-2
PNYKLTYFNLRGR	3	TY, YF, LT	6-7, 7-8, 5-6
LTYFNLR	3	TY, YF, LT	2-3, 3-4, 1-2
YLFAYAGIK	3	AY, YA, YL, CG	4-5, 5-6, 1-2
GCGNSTAGGAGGR	4	CG, CGN, GC, GA	2-3, 2-4, 1-2, 9-10
MGCGNSTAGGAGGR	4	CG, CGN, GC, GA	3-4, 3-5, 2-3, 10-11
AAPK	1	PK	3-4
AAPKD	1	PK	3-4
KSEGLPSECGSVR	1	CG	9-10
QMAGLTKEIS	1	LT	5-6
TYPTTI	1	TY	1-2
AMRMKEYPDYK	1	DYK	9-11
RLGAEWKLLSEAEK	1	GA	3-4
RPFIDEAK	1	EAK	6-8
MKEYPDYK	1	DYK	6-8
EYPDYK	1	DYK	4-6

The potential of the antioxidative peptides derived from collagen hydrolysates (ultrafiltrate fraction) were predicted

by Peptide Ranker http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php tool. It is a web-based server to predict the probability of biological activity of a given peptide sequence. Peptide Ranker tool provides the peptide score in the range of 0 to 1. The maximum score represents the most active and the least score denotes the least active peptides. All peptides with scores >0.5 were considered to be bioactive.

Peptide ranking based on bioactivity: A total of 29 peptides were evaluated for their predicted bioactivity using the Peptide Ranker tool. The results were visualized in the form of a heatmap, highlighting the distribution of bioactivity scores across all peptides. The Peptide Ranker assigns a probability score (ranging from 0 to 1), where higher scores indicate a greater likelihood of bioactivity. A commonly accepted threshold in literature for bioactive peptide prediction is 0.7 (Mooney et al. 2012; Kumar et al. 2021). (Figure 4)

Peptides such as VWF (0.976), PWTWPWT (0.970), and WPWNA (0.959) exhibited the highest bioactivity probabilities, suggesting they are strong candidates for functional roles, potentially including antioxidant, antimicrobial, or enzyme-inhibitory activities. These high scores are consistent with trends observed in in silico peptide screening studies, where short peptide sequences rich in hydrophobic or aromatic amino acids tend to show strong predicted bioactivity (Udenigwe and Aluko, 2012).

A moderate range of peptides, including CSRFHLCCK (0.913) and NGFCAFLK (0.873), also showed substantial bioactivity predictions, implying they may contribute functional roles depending on context-specific activity. On the other hand, peptides such as GCLPISK (0.518) and LTYFNLR (0.522) scored well below the 0.7 threshold, suggesting lower bioactivity potential, and may serve as negative controls in downstream in vitro assays.

This heatmap visualization aids in stratifying peptides by their bioactivity scores, allowing for a prioritization strategy in experimental validations. Similar heatmap approaches have been employed in peptide discovery pipelines to efficiently rank and compare candidates (Zielińska et al. 2017; Jin et al. 2020).

Moreover, bioinformatics platforms like Peptide Ranker, when used in conjunction with docking and stability models, have proven reliable in the pre-screening of multifunctional peptides (Dai *et al.* 2021). Thus, the top-ranked peptides identified here should be further investigated through molecular docking and in vitro assays to confirm their functional properties. Overall, the peptide heatmap presents a clear and effective method for comparing predicted bioactivity across sequences and provides a rational foundation for selecting top candidates for further biological or functional characterization.

CONCLUSIONS

The present study demonstrated that the integration of ultrasound-assisted enzymatic hydrolysis with ultrafiltration is an efficient and sustainable strategy for the valorization of chicken processing by-products into bioactive collagen hydrolysates. The optimized process produced a low-molecular-weight peptide fraction (<10 kDa) with a significantly higher degree of hydrolysis, protein content, and antioxidant activity than the untreated control and ultrasound-assisted enzymatic hydrolysate alone. SDS-PAGE analysis confirmed extensive collagen degradation into smaller peptides, while LC-ESI-QTOF-MS/MS enabled the identification of numerous peptide sequences enriched with hydrophobic and aromatic amino acids known to contribute to antioxidant functionality. Subsequent *in-silico* analyses using BIOPEP and Peptide Ranker identified several peptides, including VWF, PWTWPWT, and WPWNA, as highly promising antioxidant candidates with strong predicted bioactivity.

The combined application of *in-vitro* antioxidant assays and *in-silico* peptide screening provided a rapid and comprehensive approach for identifying functionally relevant peptides, thereby reducing the need for extensive experimental screening. Overall, this study highlights the potential of poultry skin, heads, and feet as economical and sustainable sources of high-value bioactive peptides and demonstrates the effectiveness of integrating green extraction technologies with advanced proteomic and bioinformatic tools. Future studies should focus on purification of the identified peptides, validation of their biological activities through cell-based and in-vivo models, and assessment of their stability and functionality in food systems to facilitate their application as natural antioxidant ingredients in functional foods, nutraceuticals, and health-promoting formulations.

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COMPETING INTEREST

The authors do not have any competing interests among themselves or others related to this research work.

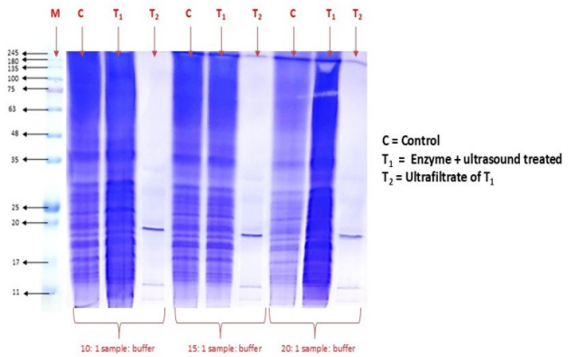

Figure 2. SDS-PAGE profile of collagen hydrolysate obtained by different treatments

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