J. Meat Sci. 2024, 19(2): 29-34

Research Article

Journal of Meat Science

Year 2024 (December), Volume-19, Issue-2

ISSN 0975-5209 (Print)
ISSN 2581-6616 (Online)

Physico-chemical and functional properties of chicken skin collagen hydrolysate: comparative effects of Collagenase and Protease

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ARTICLE INFO

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Received 21-05-2024; Accepted 22-07-2024

DOI: 10.48165/jms.2024.19.02.05

ABSTRACT

The present study aimed to utilize chicken skin as a source of collagen hydrolysate (CH) and to evaluate the impact of Collagenase and Protease enzymes on its physico-chemical and functional qualities. Collagen hydrolysate was prepared using both conventional methods (acid and alkali treatments, water bath hydrolysis) and enzymatic treatments with Collagenase (CE) and Protease (PE) at a concentration of 1:1000 w/v. A process intervention for enzymatic extraction was attempted by reducing the processing time. Enzymatic extraction significantly increased the yield of hydrolysates as compared to the conventional methods of extraction. The degree of hydrolysis (DH) indicated that enzymatic extraction enhanced the hydrolysis of collagen, with Protease (PE) showing the highest DH at 72.9%, followed by Collagenase (45.8%) and conventional extraction (31.8%). Enzymatic extraction resulted in superior functional properties with enhanced solubility, emulsifying properties, foaming properties and water and oil holding capacities, which are essential for various food and industrial applications. These improved functional characteristics suggested that enzymatic pretreatment, particularly with Protease, can be an effective method for producing high-quality collagen hydrolysates from chicken skin.

***Key words*:** Collagen hydrolysate, Enzymatic extraction, Degree of hydrolysis, Functional properties

INTRODUCTION

Poultry production accounts for 35% of the world's meat production and is projected as the livestock segment with the highest growth rate until 2050 (Li 2022). Thus, the impact of poultry waste on the environment is growing along with the soaring production numbers. In addition to chicken meat production, diverse slaughterhouse by-products are obtained during the processing of poultry. Hence, reutilization strategies to revalorize these materials have been attempted (Mandal et al., 2011) and encouraged to augment income and generate employment while addressing National and International initiatives for achieving Sustainable Development Goals. Poultry skin is one of the most abundant non-hazard protein-rich by-products generated in the meat industry (Alfonso et al., 2022). Poultry

skin has high levels of hydrophobic amino acids and acidic amino acids viz, tyrosine, lysine, histidine, glycine, alanine, glutamate, asparagine, and proline (Soladoye et al., 2015). Chicken skin proteins can be recovered in different forms, including collagen (Cliché et al., 2003), gelatin (Sarbon et al., 2013; Chand et al., 2021), protein hydrolysates (Fallah-Delavar & Farmani, 2018), and bioactive peptides (Sarbon et al., 2018). Collagen hydrolysate is a collection of peptides with a low molecular weight that can be produced by an enzyme in either an acidic or alkaline environment at a particular incubation temperature. Enzymatic hydrolysis is considered safer, cheaper, and more environment-friendly compared to chemical treatments such as acids and alkalis (Hong et al., 2019). Heating above 40 ˚C leads to denaturation of native collagen resulting in the formation of three chains in their randomly coiled shape. Once the chains are separated, hydrolysis is carried out by the action of proteolytic enzymes. The physicochemical and functional properties of the hydrolysates are majorly affected by the type of enzyme used and hydrolysis condition (e.g., hydrolysis pH, temperature, duration, and ionic strength) (Udenigwe & Aluko, 2011). To the best of our knowledge, no studies reported a comparative performance of enzymes for hydrolysate production from chicken skin.

Bearing these in mind, the present study was aimed to analyse the comparative efficacy of traditional method and enzyme-assisted methods (Collagenase type I from *Clostridium histolyticum* and Protease from *Bacillus licheniformis*) on the yield, degree of hydrolysis, and functional attributes of collagen hydrolysates derived from chicken skin.

MATERIALS AND METHODS

Indbro slow-growing broiler chicken (50 days old) was slaughtered at the Poultry Processing Plant of the Institute as per standard protocols and the skins were collected. The skin samples were scalded (55°C for 1 min), defeathered, cleaned, and packed in low-density polyethylene bags. Samples were frozen at -18°C and prior to analysis, were thawed at refrigeration temperature (4 ± 1 °C) overnight. Chemicals and all auxiliary reagents were of analytical and chemical purity.

Extraction of collagen hydrolysate (CH)

Conventional method of extraction

The skin samples were treated with 0.1N HCl (1:4 w/v) for 3 hrs at room temperature, washed with distilled water, and filtered with a muslin cloth. The acid-treated samples were then treated with 0.1 N NaOH solution (1:4 w/v) for 3 hrs at room temperature, washed, and filtered through muslin cloth again until the pH became neutral. Collagen hydrolysate was prepared by hot water extraction method in a water bath maintained at 60 °C for 6 hrs with intermittent shaking.

Collagen extraction with enzyme

Similar to the conventional methods of extraction, chicken skin samples were treated with 0.1N HCl and 0.1N NaOH washed and filtered using a muslin cloth until the pH of sample reached 7.5 – 8, followed by addition of enzyme Collagenase and Protease (1:1000 w/w) in the mix to start the hydrolysis. After 3 hrs of hydrolysis at 40 °C with Collagenase and 60 °C with Protease under constant stirring, the temperature was gradually raised to 90 °C for 10 min to inactivate the enzyme. The enzyme:substrate ratio, pH, and temperature of enzyme activity were optimized in the previous studies (unpublished data).

The resulting hydrolysate (Control, traditional method; CE, Collagenase enzyme treatment and PE, Protease enzyme treatment) was filtered using Whatman filter paper No. 42 to remove the fat layer. All the hydrolysates (C, CE and PE) were freeze-dried (0.001 Pa vacuum at -58 °C) using a laboratory scale freeze dryer (IG-LZ20, IGENE LABSERVE, Ahmedabad, India) for 36 hrs. The dried hydrolysates were obtained and are referred to as freeze dried collagen hydrolysate.

Yield

The yield of collagen hydrolysates (CH) was calculated by weighing the lyophilized collagen hydrolysate produced as a percentage of substrate used for the hydrolysis process according to Noman et al. (2018) by using the following equation:

$$\text{Yield}(\%) = \left(\frac{\text{Weight of CH}(g)}{\text{Weight of raw material}(g)} \right) \times 100$$

Degree of Hydrolysis (DH)

The degree of hydrolysis was assessed by following the method described by Maheswarappa et al. (2023) with some modifications. Around 2 ml aliquot of the aqueous suspension of hydrolysates was 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. The percentage DH was expressed as follows:

$$\text{DH } \% = \frac{\text{PC of hydrolysate} - \text{PC of supernatant}}{\text{PC of hydrolysate}}$$

Where:

PC = Protein concentration

Functional Properties of Collagen Hydrolysates

Solubility

The solubility of freeze-dried collagen hydrolysates, as a function of pH, was determined by the method described by Khiari et al. (2014) with slight modifications. About 400 mg of each CH sample homogenized in 30 ml of distilled water. Five ml of each sample was transferred to a glass test tube and the pH was adjusted to 2, 4, 6, 8 and 10 with either 0.1 M NaCl or 0.1 M NaOH and incubated at room temperature for 30 min with intermittent stirring. Each sample was centrifuged at 10,000 g for 15 min at 5 °C. The protein content of the supernatant was determined using the Biuret assay and bovine serum albumin as a protein standard. The solubility of the hydrolysates was calculated as follows:

$$\text{Solubility}(\%) = \left(\frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}}\right) \times 100$$

Foaming capacity and foam stability

Foaming capacity (FC) and foam stability (FS) were measured according to the methods of Noman et al. (2018) with some modifications. Five mg of the CH samples were mixed with 5 mL of distilled water and dissolved at room temperature. The foam was prepared using a homogenizer at 20,000 rpm for 2 min. The foam capacity was noted immediately after 2 min. Foam stability was determined by measuring the fall in the volume of the foam every 5 min for 30 min.

Foaming capacity and foam stability were calculated as follows:

$$\text{Foaming capacity} = \left(\frac{V1 - V2}{V1}\right) \times 100$$

Where:

V1 = Volume before whipping

V2 = Volume after whipping

$$\text{Foam stability} = \left(\frac{\text{Foam volume after time}}{\text{Initial foam volume}}\right)$$

Emulsifying properties

Emulsifying activity index (EAI) and the emulsion stability index (ESI) were determined using the methods described by Noman et al. (2018) with slight modifications. About 100 mg of hydrolysates was mixed with 3.335 ml of sunflower oil and 10 ml of distilled water, followed by adjustment of pH to 2, 4, 6, 8 and 10 and homogenization at 20,000 rpm for 1 min. About 50 µL was taken from the bottom of the emulsion and diluted with 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the solution was measured at 500 nm after 0 and after 10 min.

EAI and ESI were calculated as follows:

$$\text{EAI}\left(\text{mg}^2/\text{g}\right) = \left(\frac{2 \text{ x} 2.303 \text{ x A0}}{0.25 \text{ x sample weight}(\text{g})}\right)$$

$$\text{ESI}(\text{min}) = \left(\frac{\text{A0}}{\text{A0} - \text{A10}}\right) \times \text{t}$$

where:

A_0 = Absorbance at 0 min at 500 nm

A_{10} = Absorbance at 10^{th} min of emulsion

t = Time

Water and oil holding capacity

Water holding capacity (WHC) and Oil holding capacity (OHC) were estimated according to the methods described by Noman et al. (2018) with some modifications as follows:

$$\text{WHC}\left(\text{mL}/100\text{mg}\right) = \frac{\text{Weight of water absorbed}}{\text{Weight of CH sample}}$$

$$\text{OHC}\left(\text{g oil}/\text{g sample}\right) = \frac{\text{Weight of oil absorbed}}{\text{Weight of CH sample}}$$

Statistical analysis

All experiments were performed in triplicate, and the results were reported as mean ± SE. The data were

analyzed by one-way ANOVA 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 DISCUSSION

Yield and Degree of Hydrolysis

The yield and degree of hydrolysis (DH) of collagen hydrolysates were presented in Table 1. Conventional treatment recorded a yield of 4.51% (weight basis), whereas Collagenase and Protease treatment increased the yield to 5.34% and 5.36% respectively. The degree of hydrolysis was a reflection of the number of peptide bonds cleaved and, therefore, the average size of the peptides present. Conventional methods with water bath treatment resulted in a degree of hydrolysis of 31.8%. Enzymatic treatment led to a significantly (P<0.05) higher degree of hydrolysis when compared with the control, whereas PE reflected highest DH% (72.9%) followed by CE (45.8%). The results from the present study suggested that enzymes could have influenced the generation of collagen peptides with different degrees of hydrolysis, which also determines other functional characteristics of the hydrolysates (Indriani et al., 2022).

Where:

CE= Collagenase treated hydrolysate

PE= Protease treated hydrolysate

Functional properties

Solubility

The pH dependent solubility of the collagen hydrolysates derived from poultry skin were depicted in Fig. 1a. Noticeably, the solubility of CE and PE was significantly (P<0.05) higher as compared to control at all pH values. Irrespective of treatments, solubility was found in pH ranges of 2-4, which decreased sharply in solubility at the pH range of 6-8, which might be due to the isoelectric point (pI) range of collagen (7-8) (León-López et al. 2019). However, the solubility of all the hydrolysates increased at pH 10. Hydrolysates with higher DH% showed higher solubility than hydrolysates with lower DH% values, which were in accordance with findings of Naqash and Nazeer (2013) who reported that degradation of proteins into smaller peptides led to higher solubility.

Foaming properties

The foaming capacity is one of the important attributes improving the sensory properties of food, including the appearance and texture. The foaming capacity (FC %) and foam stability (FS %) of protein hydrolysates were depicted in Table 1 and Fig. 1b. Foaming properties of proteins were determined by the ability of surface-active components to absorb at the air-liquid interface and to reduce surface tension (Noman et al., 2018). Collagenase treatment significantly (P<0.05) improved the FC% (86%)

Table 1: Yield, degree of hydrolysis (DH), oil and water holding capacity, and foaming capacity of chicken skin collagen hydrolysates

	Yield (%)	DH %	OHC (g oil/g sample)	WHC (mL/100mg)	FC (%)
Control	4.51±0.25	31.8±2.51^{a}	2.83±0.10^{a}	0.39±0.04^{a}	76.0±3.05^{a}
CE	5.34±0.29	45.8±1.32^{b}	4.01±0.43^{b}	0.73±0.10^{a}	86.0±2.00^{b}
PE	5.36±0.13	72.9±1.48^{c}	3.85±0.33^{ab}	3.09±0.42^{b}	83.0±1.15^{ab}

Mean ± SE in a row with different superscripts differs significantly at P <0.05.

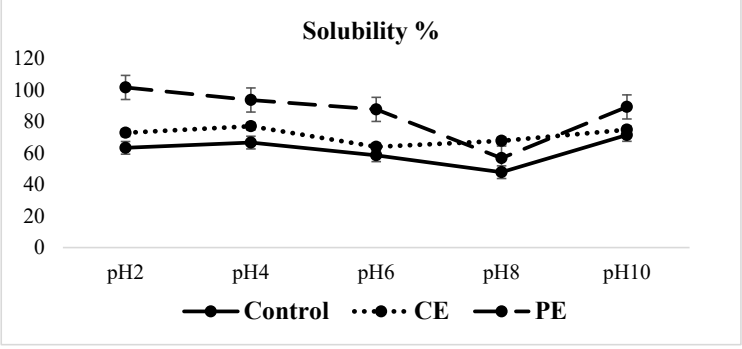

Fig. 1a: Solubility of chicken skin collagen hydrolysates at different pH conditions

followed by Protease (83%) and control (76%). Compared to Collagenase treatment or traditional method, foam stability index (%) was significantly (P<0.05) higher with Protease treatment, which maintained the FS % to more than 74.99% even after 30 min. The foaming properties of hydrolysates depends on the hydrolytic enzymes used; a significantly lower foaming stability of bovine milk protein isolate was reported with an increased degree of hydrolysis (Ryan et al., 2023).

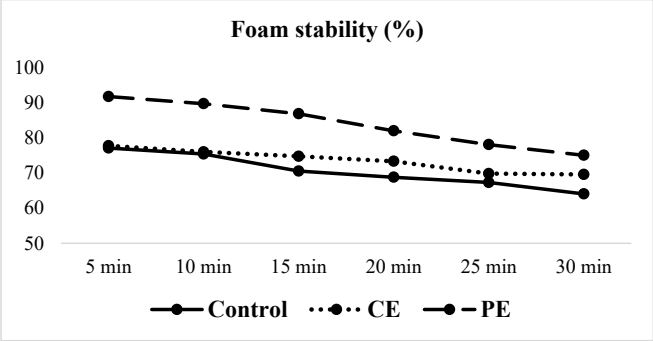

Fig. 1b: Foam stability (%) of chicken skin collagen hydrolysates

Emulsifying properties

The emulsifying property of hydrolysates in the present study was measured by the emulsifying activity index (EAI) and emulsifying stability index (ESI). EAI (mg^2/g) measures the ability of per unit protein to emulsify the maximum amount of oil, whereas ESI measures the resistance of the emulsion over a specific time (Li et al., 2019). While determining the effect of pH on emulsifying properties, the EAI and ESI of hydrolysates were found to follow the same trend as the solubility (Fig. 2). The lowest EAI and ESI were recorded at pH 6-8, since the lowest solubility was also observed at the same pH range. The hydrophilic-lipophilic balance of soluble proteins was affected by pH, which in turn determines their emulsifying activities (Chen et al., 2012). The hydrolysates obtained with Protease showed significantly (P<0.05) higher EAI than Collagenase treatment and control, which was contradictory to Chen et al. (2012), who reported that the emulsifying properties of hydrolysates in general decrease with increased degree of hydrolysis, however, the lowest ESI (min) value was reported with Protease treatment.

Oil-holding and water-holding capacities

Oil holding capacity (OHC) is an important parameter that affects the emulsifying properties of proteins and significantly influences the sensory attributes of food products. The stability of the emulsion in a food system is dependent on the binding of the fats or oils to the protein component (Biswas and Sit, 2020). The significantly (P<0.05) higher OHC (g oil/g sample) in Collagenase-derived hydrolysates might be responsible for its higher emulsion stability. Collagen hydrolysates derived from Protease treatment showed significantly (P<0.05) higher WHC (mL/100mg) than other treatments.

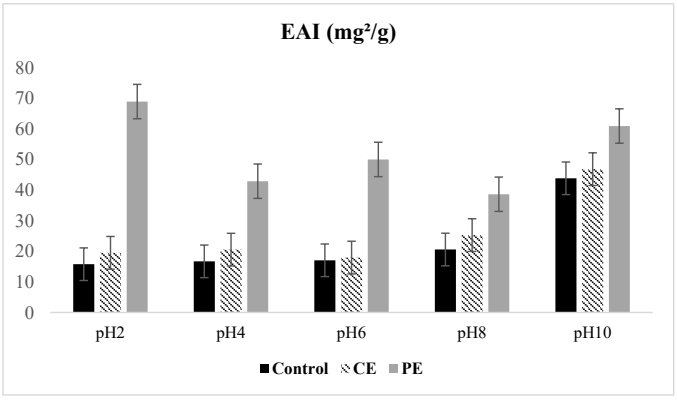

Fig. 2a: Emulsion activity index (m^2g) of skin collagen hydrolysates at different pH

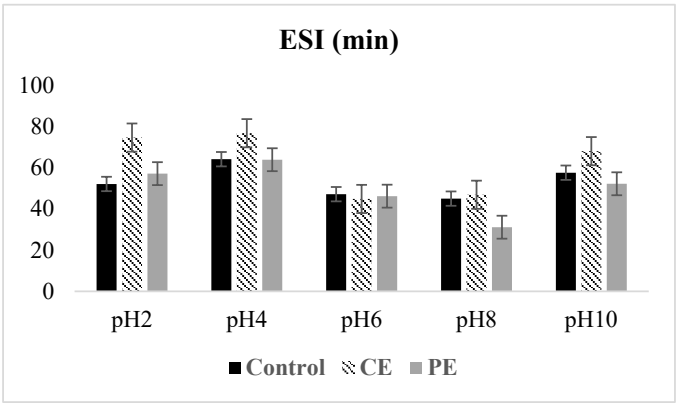

Fig.2b: Emulsion stability index (%) of skin collagen hydrolysates at different pH

CONCLUSION

The results demonstrated that collagen hydrolysates can be effectively derived from chicken skin using Collagenase and Protease hydrolysis. Although the yield of hydrolysate was not affected by the enzyme treatment; the degree of hydrolysis, solubility, WHC, emulsion activity index, and foam stability of hydrolysates were found higher with Protease treatment. This approach not only increased the yield of hydrolysates compared to conventional treatment, but also enhanced the degree of hydrolysis and functional properties. Hence, enzymatic extraction can be opted as a promising alternative to conventional methods, making them suitable for various applications in the food and industrial sectors.

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