Valorization of chicken slaughterhouse by-products: Production and properties of chicken trachea hydrolysates using commercial proteases

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**ABSTRACT**
Collagen is an abundant structural protein found in many organs of animals, for example skin, bones, and connective tissues. Chicken trachea is a collagen-rich fraction of chicken offal that is converted to a solid waste stream during poultry processing. Collagen hydrolysates and peptides have unique biological properties, which is potentially converted from chicken trachea and can be used as a useful functional ingredient. Fresh chicken trachea was sterilized by autoclaving at 121°C for 30 min and dried using freeze drier to obtain chicken trachea flakes (CTF). CTF had 68.56% protein with the 50% solubility at pH 6–10. The enzymatic hydrolysis of chicken trachea was performed using Alcalase®, Flavourzyme®, Protamex®, and Papain under the optimal condition of each enzyme. An improvement in solubility was observed. Chicken trachea hydrolysates (CTH) with the highest solubility were detected in samples hydrolyzed with Alcalase®, follow by Protamex®, Papain, and Flavourzyme®, respectively. Within an hour of hydrolysis, the CTH obtained from Alcalase® hydrolysis had the highest total amino acid contents. The top three amino acids found in this hydrolyzate powder were Glutamic acid (Glu), Glycine (Gly), and Aspartic acid (Asp). Moreover, this sample exhibited the greatest antioxidant and bioactive properties as shown by the highest antioxidant capacity from DPPH and FRAP assay (4.42 TEAC µMol/mg CTH and 22.48 TEAC µMol/mg CTH, respectively) and the lowest IC50 of ACE I inhibitor (0.41 mg/mL). These results suggested that Alcalase® hydrolysis provided the chicken trachea collagen hydrolyzate with unique properties that could be used in various food systems.

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**Introduction**

Poultry is one of the top 10 exported agricultural products of Thailand and contributes 11% of the Thai GDP about 110,484 million baths in 2020. Moreover, the increase in investment was reported at 1.5% to 2% every year.[1] Wastes and by-products from chicken production line are the most important problems for Thai chicken industries. Chicken slaughterhouses produce several solid and liquid waste streams. Solid wastes were head, bone, feather, or offal, which were filled in land or used as an ingredient of fertilizer, while liquid was blood and cleaning water. It was treated before being sent out to the environment. Poor waste management in chicken slaughterhouse leads to pollution problem and pestilence problems.[2] Over the past decade, research on liquid waste has provided opportunities for manufacturers to valorize such waste, which has resulted in improved economic and environmental sustainability of the process. However, the solid wastes have largely been overlooked. The industries start to embrace the idea of waste management, which is not just for disposal, but requires innovative solutions that are cost-effective, minimize landfill disposal and greenhouse gases, and incorporate recycling and reuse.[3]

Windpipe or trachea is a key organ of most air-breathing animals with lungs. It is a vertical tube made of rings of cartilage connecting the pharynx and larynx to the lungs, allowing the passage of air. Cartilage contains specialized cells called chondrocytes that produce a large amount of collagenous extracellular matrix, abundant ground substance that is rich in proteoglycan and elastin fibers. Chicken trachea is a part of the solid waste from slaughterhouses.[4] One problem with using chicken trachea for the production of functional peptides is that they are highly contaminated with microorganisms. High-temperature-long-time sterilization is the preferred thermal process, as it eliminates all forms of virus, bacteria, yeast, and fungi. Moreover, under intense heat treatment collagen undergoes thermal hydrolysis to yield peptides and amino acids.[5]

Currently, enzymatic hydrolysis with food-grade proteases is preferred to produce protein hydrolysate because it provides nontoxic by-products and the product composition and quality is easily controlled.[6,7] Proteases can be classified into two groups including endopeptidase and exopeptidase. Endopeptidases are proteolytic peptidases that break peptide bonds of non-terminal amino acids, while exopeptidases break peptide bonds from end-pieces of terminal amino acids. Therefore, endopeptidases cannot break down peptides into monomers, while exopeptidases can break down proteins into
monomers. The important factors that should be controlled and optimized are enzyme-to-substrate ratio, hydrolysate temperature, hydrolysis time, and pH value. Peptide is a chain of amino acids joined by peptide bonds to form a single molecule. There are 20 naturally occurring amino acids and, like letters into words, they can be combined into an immense variety of different molecules. When a molecule consists of 2–50 amino acids it is called a peptide, whereas a larger chain of >50 amino acids generally is referred to as a protein. The function that a peptide carries out is dependent on the types of amino acids involved in the chain and their sequence, as well as the specific shape of the peptide. In the human body, peptides are found in every cell and tissue and perform a wide range of essential functions. Maintenance of appropriate concentration and activity levels of peptides is necessary to achieve homeostasis and maintain health. These substances are essential proteins for human life that provide a wide variety of health promoting benefits consisting of antioxidants, antimicrobials, antioxidants, antihypertensives and anti-aging. Protein hydrolyzate might be an alternative approach for adding value to waste from poultry industry, which is an attractive ingredient.

This research investigated the appropriate enzymatic hydrolysis condition for producing functional and bioactive hydrolysates from chicken trachea was studied. Chemical, antioxidant, and bioactive properties of chicken trachea hydrolyzate were demonstrated. The molecular weight of the produced hydrolyzate was also revealed using SDS-PAGE.

**Materials and methods**

**Materials**

Chicken trachea was obtained from a chicken slaughterhouse in Bangkok, Thailand, and frozen for further experiment. The four commercial proteases used in this study were Alcalase®, Flavourzyme®, Protamex®, and Papain. Alcalase®, Flavourzyme®, Protamex® from Novozyme were purchased from local ingredient supplier in Thailand (Brenntag Co. Ltd, Bangkok), while papain from papaya latex were obtained from Sigma-Aldrich (St. Louis, MO). Disodium tetraborate, o-phthalaldehyde, Dl-dithiothreitol, SDS, and serine were purchased from Sigma-Aldrich (St. Louis, MO). Precast Gels (4–15% stain), sample loading buffer, Coomassie Bryant blue stain, and molecular weight standard for SDS-PAGE were purchased from Bio-Rad (USA). The reagent including 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), and dipeptidyl carboxypeptidase extracted from rabbit lung were obtained from Sigma-Aldrich (St. Louis, MO).

**Raw material preparation**

Frozen chicken trachea was thawed and cleaned in cold tap water. Three hundred grams of the sample were mixed with distilled water at a ratio of 1:2 (w/w), sterilized at 121°C for 30 min by autoclave, and then cooled to room temperature. The blended sample was dried using a freeze dryer (give make and model) to obtain chicken trachea flake (CTF). The CTF was frozen and stored at −20°C until required for further analysis of protein and fat content according to the AOAC method and protein solubility using the Hartree-Lowry assay.

**Enzymatic hydrolysis**

CTF was subjected to hydrolysis using the conditions shown in Table 1. Protein hydrolyzate is produced according to the method previously described by with some modifications. The CTF was mixed with distilled water and adjusted to the optimum pH of the enzyme (see Table 1) using 0.1 N NaOH. The reactions were performed in 50 mL tube with controlled temperature using a Shaking water bath (Linear Shaking Water Bath, Grant Instruments). In order to inactivate the protease enzyme, the mixture was heated at 100°C for 15 min in hot air oven. After that, the sample was
cooled down at room temperature and centrifuged at 2,010 g at 4°C for 30 min (Avanti Centrifuge, J-26 XP). The supernatant was collected and freeze-dried at −50°C, under vacuum (Edwards, Supermodulyo Freeze Dryer) to obtain chicken trachea hydrolyzate (CTH) from each enzyme, and finally it was stored at −20°C until further analysis.

**Protein content**

The nitrogen content of the sample was analyzed using CHN determination (Exeter Analytical, Hygroscopic Model 440). The crude protein content in dried chicken trachea hydrolyzate was calculated using equation (1) from Kjeldahl method.\(^{[16]}\)

\[
\% \text{ Protein content} = \% \text{ Nitrogen content} \times F
\]

where, \( F \) is conversion factor (i.e., 6.25).

**Degree of hydrolysis**

The OPA method was used to monitor the degree of hydrolysis (DH) by monitoring the reaction of o-phthalaldehyde (OPA) with the amino groups produced during enzymatic hydrolysis. Protein hydrolyzate solution (400 µL) was added to 3 mL of OPA reagents and mixed with vortex for 5 sec. The mixture was left at room temperature for 2 min before measuring the OD at 340 nm using a Genesys 10s UV-vis spectrophotometer (Thermo Scientific, UK). The DH was calculated using equation (2) to (4):

\[
\text{DH} = \frac{h}{h_{\text{tot}}} \times 100 \\

h = (\text{serine-NH}_2 - \beta) / (\alpha \text{ mequiv/g protein})
\]

\[
\text{serine-NH}_2 = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times \frac{0.9516^{245} + 0.1 \times 100}{\text{OD}_{\text{sample}}/\text{protein sample}}
\]

The value for constants \( \beta, \alpha, \) and \( h_{\text{tot}} \) were 0.40, 1.00, and 7.6, respectively, as suggested by.\(^{[17]}\)

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was performed to reveal the molecular weight profile of protein hydrolyzate. SDS PAGE precast gel (4–20%, Mini-Protean Gels, Bio-Rad) was used to analyze the protein hydrolyzate from chicken trachea. SDS PAGE was conducted under reduced conditions using a Mini-PROTEAN Tetra cell, Coomassie Brilliant Blue R-250 (Sigma) was used to stain the gels, and Plus2 Prestained (See Blue) was applied as standard marker. The electrophoretic analysis was performed using Mini-PROTEAN Tetra Cell electrophoresis apparatus (Bio-Rad Laboratories Inc., Hercules, CA) at a constant voltage of 120 V for 40 min.\(^{[18]}\)

| Table 1. Conditions for the enzymatic hydrolysis of different proteases. |
|------------------------------------------------|
| **Factor** | **Unit** | **Symbol** |
|------------|----------|------------|
| Enzymes    |          | AL         |
| Solid/Liquid ratio | % w/v | FL         |
| Enz./Subs. ratio | % w/w protein | PT         |
| pH         | pH       |            |
| Temperature | °C      | PA         |
| Time       | hour     |            |
Protein Solubility at different pH

Ten milligrams of samples were dissolved in 10 mL distilled water in plastic tube Falcon tubes. Ten CTH solution samples were adjusted to pH 2, 4, 6, 8, 10 with 0.1 NaOH or 0.1 N HCl. The suspension was centrifuged at 9,660 g for 10 min using a high-speed microcentrifuge (Benchmark, MC-12”) and the supernatant was collected. The protein content of the supernatant was analyzed using the Hartree-Lowry assay. The percentage of protein solubility in CTH was performed using a standard curve of BSA.[19]

Antioxidant properties

For all assays, freeze-dried protein hydrolyzate samples hydrolyzed by AL, FL, PT, and PA were dissolved (10 mg/mL) in phosphate buffer except in the FRAP assay where distilled water was used to dissolve the samples.

DPPH radical-scavenging activity

The scavenging effect of CTH samples or the standard Trolox for DPPH radicals was monitored according to the method described in a previous report.[20] Briefly, 500 µl of test sample was added to 500 µl of 0.1 mM DPPH ethanolic solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark, and then its absorbance was read at 517 nm using a Genesys 10s UV-vis spectrophotometer (Thermo Scientific, UK). The ability to scavenge the DPPH radical was calculated using the following equation.[5] A standard curve was prepared using Trolox in the range of 5–50 µM. The DPPH activity of CTH was expressed as µmol Trolox equivalents (TE)/mg CTH.

\[
\text{\% DPPH scavenging activity} = 1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \times 100
\]  

(5)

where, \(A_{\text{control}}\) is the absorbance of DPPH solution without sample, \(A_{\text{sample}}\) is the absorbance of DPPH solution plus test sample, and \(A_{\text{blank}}\) is the absorbance of the phosphate buffer plus ethanol.

ABTS radical-scavenging activity

ABTS radical scavenging activity of samples or Trolox was carried out using the method described previously.[21] ABTS radical solution was prepared by mixing 10 mL of 7 mM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and 5 mL 2.45 mM potassium persulfate and incubating for 15 h at 37°C. ABTS (900 µl) was mixed with 100 µL of sample and held for 6 min. The control was prepared by adding 100 µL of phosphate buffer instead of sample. For the blank, buffer was added instead of ABTS. Absorbance of the reaction mixture was observed at 734 nm using a Genesys 10s UV-vis spectrophotometer (Thermo scientific, UK). Scavenging activity was calculated using the following equation:

\[
\text{\% ABTS scavenging activity} = 1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \times 100
\]  

(6)

A standard curve was prepared using Trolox in the range of 20–200 µM. The activity was expressed as µmol Trolox equivalents (TE)/mg sample.
**FRAP radical-scavenging activity**

Ferric reducing ability (FRAP) method was applied to measure the ferric ion reducing capacity of chicken trachea hydrolysates.\(^{22}\) The model is based on the change of absorbance at 595 nm, 37°C to detect the formation of the complex tripuridiltriazine (TPTZ)–Fe(II) in the presence of reducing agents. All hydrolysates were dissolved in distilled water. Absorbance was read after incubating for 30 min using a Genesys 10s UV-vis spectrophotometer (Thermo Scientific, UK). A standard curve of FeSO₄ 7H₂O, which related to the concentration of Trolox (50–200 μM) to the absorbance at 595 nm, was performed. The results were expressed as Trolox equivalents (TE)/mg sample.

**Antihypertensive properties using ACE inhibitor**

CTH samples possessing the highest antioxidant activity obtained from each enzyme were selected for measurement of ACE inhibitor activity according to the method of\(^{23}\) with slight modifications. The samples and enzymes were dissolved in 50 mM HEPES–HCl buffer containing 300 mM NaCl (pH 8.3). Thirty microliters of CTH (0.1, 0.25, 0.5, 0.75, 1 mg/mL) were mixed with ACE solution (8 mU/50 mL, 50 μL). When the mixture was pre-incubated at 37°C for 5 min, then the 50 μl of hippuryl-L-histidyl-L-leucine (HHL, 6 mg/mL) solution were added into the mixture to initiate the reaction and incubated at 37°C for 15 min. Three hundred and eighty microliters of 1 M HCl were added to terminate the enzymatic reaction. The hippuric acid formed was extracted with 1.5 mL of ethyl acetate, and the mixture was vigorously mixed for 1 min, followed by centrifugation at 9,660 g for 10 min using a high-speed microcentrifugation (Benchmark, MC-12™). The supernatant (1 mL) was collected and heated in boiling water to evaporate the solvent and the hippuric acid retained was dissolved in 1 mL of distilled water and the absorbance read at 228 nm. The ACE-inhibitory activity (%) was determined using the following equation.

\[
\% \text{ ACE inhibitor activity} = 1 - \frac{A_{\text{sample}} - A_{\text{sample blank}}}{A_{\text{control}} - A_{\text{control blank}}} \times 100 \tag{7}
\]

where \(A_{\text{control}}\) is the absorbance of 50 mM HEPES–HCl buffer containing 300 mM plus NaCl (pH 8.3) without a sample, \(A_{\text{sample}}\) is the absorbance of 50 mM HEPES–HCl buffer containing 300 mM plus NaCl (pH 8.3) with a sample, \(A_{\text{control blank}}\) and \(A_{\text{sample blank}}\) are the control solution was added into the reaction without terminating the reaction with 1 M HCl. The IC₅₀ value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

**LC/MS methodology for Determination of Peptide Mass Distribution**

The samples were reconstituted at 10 mg/mL in 95% ACN/5% MiliQ water (18.2 Ω) and further diluted down to 1 mg/mL using the same buffer system. The chemical profiling was performed on a Dionex UltiMate 3000 UHPLC system (Thermo, Hemel Hempstead, UK) connected to a Thermo Scientific Q-Exactive Basic Hi-Resolution mass spectrometer system (Thermo, Hemel Hempstead, UK).

The chromatographic separations were achieved using a Waters T3 high strength silica (HSS) C18 UHPLC column (150 mm x 1.8 mm ID, 1.7 μm particle size) (Waters, Elstree, UK) with a flow rate of 400 μL/min operating at 45°C. The LC buffers were a binary solvent system consisting of Buffer A (Ultrapure water 18.2 Ω x ≤ 1 ppb Total organic content (TOC) with 0.1% formic acid) and Buffer B consisted of Optima grade acetonitrile with 0.1% formic acid. The LC profile was as follows: 0 min (5% B) hold for 1 min, the n process with increasing linear gradient to 100% B at 11 mins (curve 5), this was held for further 3 min (wash period) and a further column stabilization time of another 3 mins at the original starting condition (5% B).

For the heated electrospray ionization (HESI) source, the electron transfer capillary temperature was set to 275°C with an applied voltage of 3.5 kV. The RF frequency of the S-lens was set to 50. The HESI’s Sheath flow rate was set to 50, the auxiliary flow set to 15, and a sweep gas set to 3 (all arbitrary
units). Finally, the HESI was operating 425°C throughout the analysis. For the MS1 profiling, the mass spectrometer was operating at 35 K mass resolution of 200 m/z with a scan rate of approximately 7 scan/s⁻¹ and a mass range of 100–1000 m/z. The automatic gain control (AGC) was set to 1e6 with a maximum injection time of 100 ms. The total run time per analytical run was approximately 20 min from injection to injection. 5 μL injection volume and the samples were held at 4°C within the autosampler module prior to and following injections.

**Total amino acid content**

Total amino acids of CTH were determined using HPLC with a fluorescence detector as described by previous study.[24] The sample was hydrolyzed with 6 N HCl and placed in a heating block at 110°C for 22 h. After acid hydrolysis, an internal standard was added to the hydrolyzate and then this was diluted with deionized water, which had been filtered with a 0.45 μm membrane filter. The filtrate was mixed using a vortex mixer with AccQ-fluor derivatization buffer and AccQ-fluor reagent to derivatize the amino acids. Then, the sample was heated at 55°C for 10 minutes in a heating block, after which it was injected onto a Hypersil Gold column C18 (4.6×150 mm,3μm) and analyzed using HPLC. Identification of the amino acids in the sample was carried out by comparing their retention times with the standards.

**Results and discussion**

**Characteristics of chicken trachea flake**

The protein and fat content of CT and CTF were not significantly different on a dry basis (Table 2) (p > .05). The total protein content of CT and CTF was 69.71% and 68.56%, and the fat content was 16.53% and 16.95%, respectively.

These results are comparable to the protein content in duck, chicken, and ostrich trachea of 66.19, 79.19, and 84.38, respectively.[25] It is clear that heat, freezing, and drying does not affect the protein and fat content of CTF because temperature fluctuations change only the protein structure but not nitrogen content.[26] Hence, the detected protein content in the sample remained high.

Protein is a major component of CTF, and the isoelectric point (pI) of the protein constituents is an important factor determining its solubility prior to enzymatic hydrolysis. CTF had a good solubility of 45–50% over the pH range 6–10, while it was lower than 45% at pH 2. The protein is insoluble at pH 4 represented the isoelectric point where its net charge is zero.[25] The results showed that the pI of CTF was close to pH 4, and for this reason, the suitable hydrolysis condition of CTF was in the range of pH at 6–10.

**Characteristics of chicken trachea hydrolyzate (CTH)**

Figure 1 presents the soluble protein released and DH of CTH obtained from the various enzyme treatments over a period of 6 hours. For all enzymes, the initial rate of soluble protein release and the increase in DH was extremely high during the first hydrolysis hour. For Protamex® and Flavourzyme®, the soluble protein content reached the highest point after 2–3 hours and then changed slightly. For Alcalase® and papain protein release, it continually remained at a constant rate between 1 and 6 hours.

| Table 2: Protein and fat contents (% dried basis) of fresh chicken trachea (CT) and chicken trachea flake (CTF). |
| Sample | Protein (%) | Fat (%) |
| CT | 69.71 ± 2.38 | 16.53 ± 0.36 |
| CTF | 68.56 ± 0.39 | 16.95 ± 0.00 |

Note: “ns” Not significant difference between mean in the same column (p > 0.05)
After 6 hours, the CTH hydrolyzed by Alcalase® had highest soluble protein content, followed by papain, Protamex®, and Flavourzyme® (Figure 1A). These results indicate the effectiveness of protein hydrolysis using commercial proteases that are suitable for commercial applications.

The DH of the CTH hydrolysates produced by these enzymes had similar trends to the soluble protein released. Initially, the DH increased sharply in the first hour, and then slowed down from 1 to 6 hours. After 6 hours of hydrolysis, the final DH of all samples was followed in the same order as for the protein released, i.e., the highest DH was found with Alcalase®, followed by papain, Protamex®, and finally Flavourzyme®. This might be because of the highest enzyme activity of Alcalase®. This enzyme gave the aggressive hydrolysis with the activity of 2.4–4.0 AU-A/g. This commercial protease was versatile endo-protease suited for animal protein extraction and provided very extensive hydrolysis. With this property, Alcalase® yielded the hydrolyzate with the highest DH.

Figure 1. Protein content and DH of CTH hydrolyzed with 4 protease (AL = Alcalase®, FL = Flavourzyme®, PT = Protamex® and PA = Papain) at different hydrolysis time (0, 1, 2, 3, 4, 5, 6 hours).
Regarding the fat content in CTF, the effect of fat from raw material on enzymatic hydrolysis was not detected. The solid-to-liquid ratio was controlled at 4% w/v. Only 4 g of dried chicken trachea flake was added into 100 mL water, hence the lipid in the sample during hydrolysis was then diluted 25 times. For each hydrolysis flask (8 g to 200 mL), it contained only 1.356 g of fat in 200 mL of water. At this concentration, the effect on enzyme activity was not detected. Moreover, there was a report of squid head protein hydrolysis that also uses commercial protease hydrolysis without fat reduction. [27]

SDS-PAGE (Figure 2) was also used to monitor the progress of proteolysis. The CTH obtained from Alcalase® and Protamex® hydrolysis showed almost complete hydrolysis after the first hour of hydrolysis, with all peptides formed having molecular weight smaller than 6 kDa. On the contrary, larger peptides were observed when Flavourzyme® or Papain enzyme was used and these persisted in protein bands for longer hydrolysis times. CTH with papain hydrolysis shows pronounced bands on the SDS PAGE gel over a wide range of molecular weights.

The CTH hydrolysis results can be compared to other protein systems hydrolyzed with the same enzymes. Hydrolysates have been produced from low-ash poultry meal using Alcalase®, Protamex®, and Flavourzyme®, giving a DH (%) at 7 h of 9.41, 10.63, and 2.54, respectively. [28] Goat milk hydrolyzed with Alcalase®, Flavourzyme®, Papain, proteinase K, and trypsin gave a significantly higher DH with proteinase K, Alcalase®, and trypsin than for Papain and Flavourzyme®. [29] Rice bran protein concentrate and soybean protein hydrolyzed with Alcalase® and Papain showed a higher DH with Alcalase®. [6] Various authors report that, when compared to other commercial protease, Alcalase® results in higher protein recovery. Shrimp waste hydrolyzed with Alcalase® showed mean protein recovery in the range of 59–60%, which was significantly (p ≤ 0.05) higher than Neutrase® and also higher than Protamex® and Flavourzyme® by 5.31% and 8.20%, respectively. [7] This result might be due to the combination of enzyme in Alcalase® which was endopeptidases. Peptides and protein

![Image](image.png)

Figure 2. SDS-PAGE analysis of CTH hydrolyzed with Alcalase® (A), Flavourzyme® (B), Protamex® (C) and Papain (D). Lane contents are as follows: M1 is Marker; M2 is Small molecule Marker; flow-through; Lane 3 is Protease; 0 H – 6 H are CTH that hydrolyzed and inactivate at 0, 1, 2, 3, 4, 5 and 6 hours.
hydrolysates received from this enzyme hydrolysis were generated fast and aggressive at the optimal pH and temperature used in this study. As a result, protein hydrolyzate with high DH and low molecular weight was observed.

**Solubility of chicken trachea hydrolyzate**

All freeze-dried powders made from the hydrolyzed CTF using any of the proteases had greater protein solubility than for the non-hydrolyzed CTF, with the solubility increasing with hydrolysis time as shown in Figure 3. Alcalase® (AL) and Protamex® (PT) created CTH, which had high solubility at pH 4 after hydrolysis for only 1 h. However, Flavourzyme® (FL) and Papain (PA) showed a more gradual increase in solubility with hydrolysis time. Enzymatic hydrolysis reduced the average molecular mass of proteins/peptides and liberates ionizable groups. Both factors improve protein solubility.[30] Collagen has a distinct composition compared to other proteins, as it is rich in glycine and proline, and does not contain cysteine and tryptophan.[5] As a result of prolonged heating in water, collagen undergoes partial hydrolysis, which occurs with structural changes, due to the breaking of inter-chain bonds, transforming into collagen hydrolyzate.[31] Results indicated that cleavage of peptide bonds was higher in enzymatic hydrolysis and led to the formation of smaller peptides and free amino acids.[20]

**Bioactive properties of chicken trachea hydrolyzate**

The antioxidant properties of CTH produced by the different proteases and at different hydrolysis times were measured using DPPH, ABTS, and FRAP assays. These methods were measured the ability of a molecule to donate hydrogen atoms to free radicals. The data of these three assays are shown in Figure 4 presented as Trolox Equivalent Antioxidant Capacity (TEAC) in μM/mL. For all samples, and

![Figure 3. %Protein Solubility at different pH of CTH hydrolysate Alcalase® (A), Flavourzyme® (B), Protamex® (C) and Papain® (D) at hydrolysis time 0, 1, 2, 3, 4, 5, 6 hrs.](image-url)
for all assays, there was an initial rapid increase in antioxidant capacity within the first hour of hydrolysis, with the curves leveling off for longer hydrolysis times. In general, Alcalase® was the best protease for producing antioxidant protein hydrolyzate from CTF (DPPH = 4.02–4.27 TEAC μM/mL, ABTS = 134.29–148.84 TEAC μM/mL; FRAP = 16.67–22.48 TEAC μM/mL) whilst Papain gave CTH with the lowest antioxidant properties (DPPH = 0.43–3.71 TEAC μM/mL, ABTS = 67.99–89.87 TEAC μM/mL; FRAP = 11.57–12.51 TEAC μM/mL) (Figure 4A–C). For Alcalase®, the optimum antioxidant capacity found in the FRAP assay after 1 hour and then started to decrease again after 3 hours.

Figure 4. Antioxidant ability of hydrolyzate from chicken trachea hydrolyzed with 4 protease (AL = Alcalase®, FL = Flavourzyme®, PT = Protamex® and PA = Papain) at different hydrolysis time (0, 1, 2, 3, 4, 5, 6 H.).
Protamex® and Flavourzyme® gave CTH with antioxidant capacity intermediate between Alcalase® and Papain, but the three assays did not give a consistent view of which of these two gave CTH with higher antioxidant capacity. It should be noted that although the three assays do, in general, give consistent trends in antioxidant capacity of the hydrolysates from the four enzymes, the absolute TEAC units for the three assays differ markedly. This is because these assays used the different theoretical basis of the chromogen formation reaction. Chemical reagents that form different chromogen could be detected with spectrophotometer at different wavelengths.\[32\] Hence, the different numbers of antioxidant capacity from these three assays resulted.

Proteins are biopolymers that consist of amino acids linked by peptide bonds that are formed between the primary carboxyl and amino groups. The other parts of the amino acids constitute the side groups that contribute to the structural and functional properties of proteins.\[33\] The interactions between the R residues of amino acids in the polypeptide chains, which can establish different types of intramolecular bonds such as: hydrogen bonds, ionic bonds, covalent bonds, van der Waals forces, dipole–dipole interactions. The reason is that peptides chains have the potential to donate hydrogen atoms that can quench free radicals; these groups could possess excess electrons that can also be used to neutralize free radicals.\[34\]

The ACE inhibitory capacity of CTH hydrolyzed by the four proteases for 1 h was measured and is presented in Figure 5 by 50% as IC_{50} i.e. the concentration at which 50% inhibition occurs. CTH itself has ACE inhibitory activity with an IC_{50} of around 1.2 mg/mL. This was improved significantly by hydrolysis of the CTH for 1 h, with a reduction in IC_{50} of around 50% or greater. Alcalase is the best enzyme for producing antihypertensive CTH hydrolyzate with an IC_{50} of 0.422 mg/mL.

A variety of antioxidant and antihypertensive peptides have been isolated from hydrolysates of foods, plants, food by-products, and from various meat proteins.\[35\] A report in 2017\[36\] investigated the production of bioactive peptide from bovine lungs using Papain, Pepsin, and Alcalase®. The researchers reported that Alcalase® hydrolysis produced hydrolyzate with the lowest IC_{50} for the
The average molecular weight (MW) of CTH hydrolyzed by Alcalase®, Flavourzyme® (FL), Protamex® (PT), and Papain (PA) for 1 h is shown in Table 3. FL gave the lowest average MW at 252.65 Da followed by Papain (283.05 Da), Protamex (411.48 Da), and Alcalase® the highest (487.43). This supports the results of SDS PAGE, which indicated that all samples contained peptides of molecular weight lower than 6 kDa after hydrolysis. Flavourzyme® and Protamex® endo and exo – peptidase activity, cleaving the protein at both terminal positions and in the middle of the protein/peptide chain, thus increasing the proportion of low molecular weight peptides faster, when compare with Alcalase®, which is endopeptidase activity.

Table 3. Average molecular weight (Da) of hydrolyzate from chicken trachea hydrolyzed with Alcalase® (AL), Flavourzyme® (FL), Protamex® (PT), and Papain (PA). Average molecular weight was calculated from HPLC/MS data for hydrolysates from four enzymes after 1 hour of hydrolysis.

| Sample | Average molecular weight (Da) |
|--------|-------------------------------|
| AL     | 487.43                        |
| FL     | 252.35                        |
| PT     | 411.48                        |
| PA     | 283.05                        |

The average molecular weight (MW) of CTH hydrolyzed by Alcalase®, Flavourzyme®, Flavourzyme® (FL), Protamex® (PT), and Papain (PA) for 1 h is shown in Table 3. FL gave the lowest average MW at 252.65 Da followed by Papain (283.05 Da), Protamex (411.48 Da), and Alcalase® the highest (487.43). This supports the results of SDS PAGE, which indicated that all samples contained peptides of molecular weight lower than 6 kDa after hydrolysis. Flavourzyme® and Protamex® endo and exo – peptidase activity, cleaving the protein at both terminal positions and in the middle of the protein/peptide chain, thus increasing the proportion of low molecular weight peptides faster, when compare with Alcalase®, which is endopeptidase activity.

Total amino acid composition of CTF and CTH after hydrolysis for 1 h by the four enzymes showed release of 15 amino acids in the protein hydrolyzate (Table 4). The most abundant amino acids in both CTF and all CTH were Glu, Gly, Ala, and Pro. Alcalase® hydrolyzate had the highest total amino acid content of 62.71 mg/100 mg, consistent with its endo-peptidase activity.

Hydrolysis enhances hydrophilic amino acids in the terminal chain of peptide and as a result improves the solubility of protein hydrolyzate. This was confirmed by an increase in hydrophilic amino acids such as serine (Ser) and threonine (Thr) when CTF was hydrolyzed by Alcalase® and Protamex®. Moreover, Glu – a charged hydrophilic amino acid and Gly – a polar amino acid was increased by enzymatic hydrolysis. The hydrophobic amino acids in the protein hydrolyzate have been reported to contribute to their antioxidant and antihypertensive properties. According to the research of Leon-Lopez, et al. (2019), the enzymatic treatment of collagen showed significant increases in aspartic acid and glutamic acid due to enzymatic cleavage of the polypeptide chains of collagen fibers. Furthermore, the protein hydrolyzate of chicken meat hydrolyzed by Alcalase® contained glutamic acid and tryptophan at 14.00% and 12.37%, respectively.

**Conclusion**

This study indicated that chicken trachea can be a source of protein for producing protein hydrolyzate. Alcalase®, Flavourzyme®, Protamex®, and Papain were effectively hydrolyzed chicken trachea to produce unique collagen hydrolysates. The Alcalase® hydrolysis for 1 hour provided collagen hydrolyzate with the best antioxidant activity. The obtained hydrolyzate had molecular weight lower than 6 kDa and had good solubility along the studied pH range. The solubility of the hydrolyzate showed an improvement over the non-hydrolyzed CTF, particularly at pH 4 (the isoelectric point of collagen). The antioxidant and antihypertensive properties showed a correlation with hydrophobic amino acid
content. In conclusion, chicken tracheas appear to be a suitable alternative source of collagen hydrolyzate compared to typical sources like bone skin and offal.

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Disclosure statement

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Table 4. Total amino acid of hydrolyzate from chicken trachea hydrolyzed with Alcalase® (AL), Flavourzyme® (FL), Protamex® (PT) and Papain (PA).

| Amino acid | CTF | AL  | FL  | PT  | PA  |
|------------|-----|-----|-----|-----|-----|
| Asp        | 5.21| 5.58| 5.25| 5.24| 5.02|
| Ser        | 2.28| 2.54| 2.28| 2.33| 2.18|
| Glu        | 10.48| 11.80| 10.90| 10.86| 10.74|
| Gly        | 6.98| 8.65| 7.69| 7.81| 8.02|
| His        | 0.71| 0.75| 0.67| 0.68| 0.62|
| Arg        | 3.58| 4.41| 3.75| 3.82| 3.66|
| Thr        | 2.28| 2.59| 2.26| 2.29| 2.13|
| Ala        | 4.38| 5.00| 4.57| 4.62| 4.53|
| Pro        | 4.86| 5.82| 5.04| 5.14| 5.11|
| Tyr        | 1.19| 1.32| 1.08| 1.13| 1.02|
| Val        | 2.32| 2.46| 2.29| 2.31| 2.06|
| Lys        | 3.66| 4.02| 3.59| 3.67| 3.38|
| Ile        | 1.77| 1.85| 1.77| 1.76| 1.54|
| Leu        | 3.72| 3.97| 3.60| 3.73| 3.34|
| Phe        | 1.84| 1.95| 1.74| 1.80| 1.63|
| Σ Amino acid | 55.26| 62.71| 56.48| 57.19| 54.98|
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