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Exploring Chicken Sternal Cartilage as an Alternative Source of Bioactive Protein Hydrolysates

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Abstract

There is a growing concern on the correct use and dispose of the by-products of the food industry, and chicken slaughterhouse, such as cartilage, combs, and wattles may be strong candidates as a protein source for bioactive peptides, however it is crucial to understand how the proteolysis process may affect the peptide profile and their bioactivity. The objective of this work was to obtain protein hydrolysates from chicken sternal cartilage using Alcalase and Flavourzyme as catalysts and to determine their antioxidant capacity and acetylcholinesterase (AChE) inhibition potential. The cartilage was hydrolyzed with Alcalase (pH 8, 50°C, 1.8 wt%$_{enzyme:substrate}$, substrate dry basis) and Flavourzyme (pH 7, 50°C; 1.8 wt%$_{enzyme:substrate}$, substrate dry basis). Alcalase demonstrated a greater capacity for cartilage digestion (97.42% yield), and the resulting cartilage hydrolyzed a 1.8-fold higher capacity for AChE inhibition than Flavourzyme-catalyzed hydrolysate. On the other hand, Flavourzyme produced a hydrolysate with a 1.3-fold higher capacity on TBARS inhibition (74.2%) than Alcalase. The hydrolysates' amino acid profiles were delineated via HPLC, revealing the presence of 18 amino acids, notably, tryptophan, tyrosine, and histidine exhibited similar concentrations across the hydrolysates. The tryptophan/LNNA (large neutral amino acids) ratio was elevated in the hydrolysate generated by Flavourzyme, potentially correlating with its heightened TBARS reduction potential. Furthermore, the increased total amino acid content observed in the hydrolysate produced with Alcalase may be linked to its efficacy in AChE inhibition.

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INTRODUCTION

The poultry industry, a rapidly expanding livestock sector, produces substantial by-products with harmful environmental effects that need revalorization. While studies propose using protein-rich slaughterhouse waste as bio-fertilizers [1] or for biomethane and biogas production, [2] this practice may not be the most cost-effective approach. The rise in non-communicable diseases worldwide, driven by modern lifestyles, has led to extensive efforts to discover new biologically active agents. Chicken slaughterhouse by-products, such as cartilage, combs, and wattles, [3] are promising due to their protein content, which can be processed to produce bioactive peptides through proteolysis [40].

Using these by-products solely as fertilizers can be considered a waste of valuable resources. Instead, repurposing them as a protein source for bioactive peptides through proteolysis increases

their added value [4]. By converting chicken slaughterhouse by-products into high-value bioactive peptides, we reduce waste and open new possibilities for applications in the health and pharmaceutical industries, offering a more profitable and sustainable use.

Hydrolyzed collagens are gaining more and more attention due to various interesting biological properties implicated in various health benefits, such as nutraceutical and nutricosmetic solutions [5]. Noncommunicable diseases (NCDs, diseases that cannot be transmitted directly from one person to another) are the primary factors affecting the health and longevity of modern humans. According to the WHO, NCDs account for 70% of the global disease burden and are responsible for approximately 70% of all deaths worldwide [6]. Specifically, hydrolyzed collagens can play a role in preventing and treating: i) Cardiovascular Diseases (can help lower blood pressure, reduce cholesterol levels, and improve endothelial function), [7] ii) Type 2 Diabetes (may enhance insulin sensitivity, regulate blood glucose levels, and improve metabolic health), iii) Obesity (can promote satiety and reduce appetite, aiding in weight management and preventing obesity-related complications), iv) Hypertension (some protein hydrolysates have been shown to possess antihypertensive properties, helping to manage high blood pressure), v) Metabolic Syndrome (the combined effects of protein hydrolysates on weight, insulin sensitivity, and lipid profiles can help manage or prevent metabolic syndrome), vi) Chronic Inflammation-Related Diseases (can help mitigate the inflammatory response linked to diseases like rheumatoid arthritis and certain cancers) and vii) Osteoporosis (can support bone health by enhancing calcium absorption and promoting bone density). Several in vivo studies have reported hydrolyzed collagens bioactivities, such as antioxidant,[8] antihypertensive, [9] and antidiabetic, [10] among other beneficial effects [10]. Chicken protein hydrolysates have already been associated with actions on inflammatory changes and oxidative stress on human dermal fibroblasts,[11] embryonic mouse fibroblast cell line NIH-3T3, [12] and mice who were high-fat diet-induced to obesity [13].

Brandão-Rangel et al. [14] explored hydrolyzed collagen consumption's effects on the skin in an *in vitro* study. The authors concluded that hydrolyzed collagen (types I and III) inhibited lipopolysaccharide-induced inflammation in skin fibroblasts and keratinocytes while improving the synthesis of pro-collagen-1α by skin fibroblasts, as well as inducing the proliferation of skin fibroblasts and keratinocytes.

The hydrolysate peptides are absorbed by the gastrointestinal tract and transferred to the blood, thereby becoming available for metabolic processes [15]. Soniwala et al. [16] proposed that agents like a natural mixture of hydrolyzed type-2 collagen and chondroitin sulfate (hCol2) influence joint degeneration indirectly through an alteration in the gut microbiome of mice. Key proinflammatory microbes that were increased in the obese mice were reduced when supplementation with hCol2 was implemented. A series of beneficial microbes were also identified in supplemented mice, including *Bifidobacterium pseudopodium*.

Another important bioactivity that has been explored to identify the potential of different substances against Alzheimer Disease (AD) is the acetylcholinesterase (AChE) inhibition capacity. AChE is an enzyme that catalyzes the hydrolysis of acetylcholine (ACh, a neurotransmitter) into choline and acetic acid. When a deficit of ACh is present in the brain, a dysfunction of acetylcholine-containing neurons occurs, contributing substantially to the cognitive decline observed in those with advanced age and Alzheimer's disease (AD) [17]. Kim et al. [18] investigated the protective effect of low molecular weight hydrolysates of pig skin (produced with Flavourzyme 1000L), against scopolamine-induced impairment of cognitive function in mice. The authors determined the acetylcholine content and acetylcholinesterase enzyme (AChE) activity in the animal's brain tissue. The authors concluded that mice treated with the hydrolysate at 400 $mg/kg_{body\ weight}$ had a significantly higher ACh content in the brains than those dosed with scopolamine. Furthermore, compared to scopolamine-treated mice (100% activity), AChE activity was reduced to 26.7% for mice treated with that hydrolysate dose.

Cartilages are interesting to be used as raw material for hydrolysates as they contain high amounts of type-2 collagen [19]. Type-2 collagen, traditionally, has been extracted from bovine or porcine articular cartilage. Still, it also can be found in a recognized potential source of type-2 collagen: the chicken sternal cartilage, a by-product of the chick manufacturing industry. The study conducted by Chen et al. [20] investigated the effects of daily consumption of type II collagen hydrolysate derived from chicken hydrolyzed cartilage in 160 patients diagnosed with knee osteoarthritis (OA) over 24 weeks. The results showed a significant reduction in pain levels after just 14 days. Additionally, combining chicken hydrolyzed cartilage with the supplement Essence of Chicken (broth commonly used in some Asian cultures, rich in nutrients, often consumed as a tonic for overall well-being) led to improvements in fat-free mass and grip strength compared to both glucosamine HCl (reference nutraceutical) and placebo after 24 weeks. These findings suggest that collagen hydrolysate may be effective in managing OA symptoms and enhancing mobility in older adults. Thus, chicken sternal cartilage can be used as raw material for high-added-value products, such as hydrolyzed collagen, instead of its conventional use to produce animal feed [21]. The main objective of this study was to assess the antioxidant capacity and AChE inhibition potential of hydrolysates derived from chicken sternal cartilage, employing Alcalase and Flavourzyme as catalysts for hydrolysis. These investigations aimed to correlate these outcomes with the amino acid profiles of the hydrolysates.

MATERIAL AND METHODS

Material

Chicken sternal cartilages were obtained in a local market in 2021 (Campo Mourão, Paraná, Brazil). Alcalase 2.4 L (≥ 2.4 U/g, P4860, Sigma-Aldrich, optimal pH and temperature of 9-10.5 and 50-60°C, respectively [22]) and Flavourzyme (Novozymes Biotechnology Co., optimal pH and temperature of 5.5-7.7 and 50-55°C, respectively [23]) enzymes were used for cartilage hydrolysis. For the acetylcholinesterase (AChE) activity evaluation the following materials were used: tri-hydroxymethyl aminomethane (*Tris*-HCl, Dinamica, P.A.), acetylcholinesterase enzyme from *Electrophorus electricus* (electric eel, Type V-S, lyophilized powder, ≥ 1,000 units/mg protein, Sigma-Aldrich), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB, 98%, Sigma-Aldrich) and acetylthiocholine iodide (ASCh) (Sigma-Aldrich, 99% (AT)). Potassium phosphate buffer (TFK) was prepared using monobasic potassium phosphate (DinamicaDinamica, P.A.) and dibasic potassium phosphate (Neon, P.A.). For the antioxidant assays, the following reagents were used: 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich, ≥ 98%, HPLC grade), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich), 2,4,6-*Tris*(2-pyridyl)-s-triazine (TPTZ, Sigma-Aldrich, ≥ 98%), Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid, Sigma-Aldrich), ferrous sulfate (Synth, P.A.), ascorbic acid (Dinamica, P.A.), trichloroacetic acid (Dinamica, P.A.) and 2 thiobarbituric acid (Exodo, P.A.). Bovine Serum Albumin (Sigma-Aldrich, 66,000 g/mol, heat shock fraction, protease-free, fatty acid-free, essentially globulin-free, pH 7, ≥ 98%) was used as a standard for the degree of hydrolysis determination. To identify and quantify the hydrolysate's amino acids (Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe and Lys) an external standard was used (Standard H, Pierce, P/N 20088), as well as an internal standard (alpha-aminobutyric acid, Sigma-Aldrich, ≥ 98%).

Chicken Sternal Cartilage Pre-Treatment and Characterization

Pre-treatment of the chicken sternal cartilage consisted of grounding in an electric meat grinder, manually homogenization (pool), and frozen at -10°C until used in the characterization analyses or in hydrolysis.

For proximate composition analysis, the gravimetric method was used to determine the moisture content at 105°C until constant weight (3 g sample). After that, the remaining sample was incinerated in a muffle furnace at 550°C for the ashes content. The Microkjeldahl method was used to obtain the protein content (1 g sample), and the lipid content was determined by the Bligh-Dyer method [24] (10 g sample).

Chicken Sternal Cartilage Hydrolysis

The methodology, as well as the optimal conditions determined and described by Moreira et al. [25] were applied to obtain the chicken sternal cartilage hydrolysate samples. For this, pre-treated chicken sternal cartilage was thawed for 12 hours at 22°C. Two different protein hydrolysates were obtained through enzymatic hydrolysis using Alcalase 2.4 L (sample nominated as HA) and Flavourzyme (sample HF). First, the samples were dispersed in distilled water (1:5, w:v). Then, samples had their pH adjusted (8 for HA and 7 for HF) using 1 M NaOH or 1 M HCl (It is worth noting that pH was monitored during the procedure and no appreciable changes were detected). Finally, each enzyme was added at 1.8 wt% (enzyme to substrate dry basis) concerning the protein content in the samples (enzyme:substrate, E:S). Thus, hydrolysis was carried out under stirring in a water bath (Dubnoff Bath, Bunker) with a temperature adjusted to 50ºC.

After 4 hours of hydrolysis, the enzymes were inactivated by heating the mixtures in the water bath at 90°C for 15 minutes. Each mixture was centrifuged at 6,000 rpm (Nova Técnica) for 10 minutes, and the supernatant was collected and filtered through a cellulose filter (80 g/m^2) with a vacuum pump (Prismatec). The hydrolysates obtained were frozen in a freezer (Consul) for 24 h and dried in a freeze dryer (Liotop L101, Liobrás) until constant weight.

Hydrolysate's Characterization

Hydrolysis yield (HY %)

It was determined in hydrolysates after thermal inactivation and cooling at room temperature. The hydrolysates were weighed (TS, mg) and then subjected to centrifugation (6,000 rpm for 20 min), in which the aqueous fraction containing the hydrolyzed protein (ASF) was transferred, weighed, and stored at −80°C. The yield was calculated according to Equation (1).

$$HY(\%) = \frac{\text{ASF (mg)}}{\text{TS (mg)}} x100 \text{ Equation} \quad (1)$$

Degree of Hydrolysis (DH %)

The methodology described by Moreira et al. [25] was applied. Immediately after hydrolysis, aliquots were removed from the hydrolysis reaction mixture (6 mL) and trichloroacetic acid was added (4 mL, 6.25%) to promote precipitation. After 15 min of rest, the samples were centrifuged at 6,000 rpm for 10 minutes. The protein contents of properly diluted samples were then measured by Lowry's method using bovine serum albumin as the standard ($y = 16.541x + 0.0238$; $R^2 = 0.9915$). Diluted samples had their absorbance read at 750 nm on a UV-Vis spectrophotometer (USB-650-UV-VIS, Red Tide Spectrometer). DH (%) was calculated as the protein in the supernatant (SP, mg) over the total protein content in the solution (TP, mg) as calculated in the proximate composition analysis (Equation (2)).

$$DH(\%) = \frac{\text{SP (mg)}}{\text{TP (mg)}} x100 \text{ Equation} \quad (2)$$

Amino Acids Profile

The method for amino acid analysis was based on Hagen et al. [26] and White et al. [27] The samples were subjected to acid hydrolysis in order to assure no protein or polypeptide chain was allowed to enter the chromatographic system. Phenyli sothiocyanate (PITC) is used for pre-column derivatization of the aminoacids which is then separated by high-performance liquid chromatography (HPLC) and detected by their UV absorbance. Although the maximum absorbance of the derivatives is 269 nm, detection at 254 nm is considered satisfactory [28]. The pre-column derivatization method is often used due to its improved sensitivity and short analysis time when compared to ion exchange chromatography [29]. The separation of amino acids was performed by reverse phase liquid chromatography (UV-1800 SHIMADZU Corporation, Tokyo, Japan) equipped with C18 reverse phase column (50ºC) (LUNA C18, 100 Å, 5 µm, 250 x 4.6 mm, Phenomenex Inc., Torrance, USA), with DAD detector at 254 nm. The

mobile phase used was a gradient composed by (A) Sodium acetate buffer 94% (0.036 M, pH 6.4) + 5.7% acetonitrile and (B) Acetonitrile 40%. Gradient time (45 minutes): In the concentration of the mobile phase A and time (min) respectively (95%, 0-5), (77.5%, 5-10), (68%, 10-12), (40%, 12-20), (0%, 20-36), (95%, 36-45), and flow rate of 1 mL min^{-1}, volume injected of 50 µL.

Antioxidant Capacity

DPPH

The methodology applied for DPPH radical scavenging activity evaluation was previously described by Brand-Williams et al., [30] with some modifications. For that, 50 µL (30 mg/mL) of hydrolysate were added to 1,950 µL of 60 µmol/L DPPH methanolic solution in a test tube, and 50 µL of ethanol to 1,950 µL of DPPH solution in another tube (negative control). The solutions were protected from light for 30 minutes, and then the absorbance was read on a spectrophotometer at 517 nm, using methanol as a blank sample. The results were obtained using the Trolox calibration curve ($y = 0.0905\ x + 0.8656$, $R^2 = 0.9984$) and expressed in µmol of Trolox equivalent per gram of hydrolysate. The experiment was carried out in triplicate.

ABTS

ABTS radical scavenging activity was evaluated following the protocol described by Thaipong et al. [31] The working solution was prepared by mixing two solutions in equal amounts (20 mL each), a solution of 7.4 µmol/L of ABTS with 2.6 µmol/L of potassium persulfate that reacted for 12 hours in the dark. After this period, 1 mL of this mixture was added to 60 mL of methanol (named working solution). An absorption reading was taken to check if the value was close to 1.10 ± 0.02 on a UV-Vis spectrophotometer at 734 nm.

For the analysis itself, 150 µL of hydrolysate (solution at 20 mg/mL) were mixed with 2,850 µL of the working solution in test tubes and stored away from light for two hours. Then, the absorbance readings of the samples were taken at 734 nm. The values obtained were compared with the Trolox calibration curve ($y = -0.0017x + 1.0678$, $R^2 = 0.9913$) and expressed in µmoL of Trolox equivalent per gram of hydrolysate. The experiment was carried out in triplicate.

FRAP

The Ferric Reducing Power (FRAP) assay was carried out according to the method described by Thaipong et al. [31] and for that stock solutions of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM, 2,4,6-tripyridyl-s-triazine) in HCl (40 mM) and $FeCl_3.6H_2O$ (20 mM) were prepared. The working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3.6H_2O$ solution and then heated to 37°C before use. Hydrolysate (solutions at 150 mL, 20 mg/mL) were reacted with 2,850 mL of FRAP solution for 30 min in the dark. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm in an UV-Vis spectrophotometer. Results were expressed as $mM_{Trolox\ Equivalent}/g_{hydrolysate}$ (Trolox calibration curve: $y = 0.0012x + 0.0008$, $R^2 = 0.9975$). The experiment was carried out in triplicate.

TBARS

The Inhibition of Lipid Peroxidation by Thiobarbituric Acid Reactive Species (TBARS) methodology applied was previously described by Ueda et al., [32] where some modifications were implemented. Rat brains, obtained from the State University of Maringá (project approved by the ethics committee with protocol (CEUA Nº 2935011018)), were dissected and homogenized with an Ultraturrax in ice-cold *Tris*-HCl buffer (20 mM, pH 7.4), to produce a 1:2 (w/v) brain tissue homogenate that was centrifuged at 3,000 g for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with different concentrations of hydrolysate (0.2 mL, solution at 30 mg/mL) in the presence of $FeSO_4$ (10 mM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37ºC for 1 h. The reaction was stopped by adding trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL)

addition, and the mixture was then heated to 80°C for 20 minutes. Afterward, it was centrifuged again at 3000 g for 10 min to remove the precipitated protein, and the color intensity of the MDA-TBA complex (malondialdehyde-thiobarbituric acid chromophore complex) in the supernatant was measured by its absorbance at 532 nm in an UV-Vis spectrophotometer. The TBARS inhibition (%) was calculated using Equation (3), where A is the control absorbance and B is the absorbance of sampled containing hydrolysate. The experiment was carried out in triplicate.

$$TBARS\ Inhibition\ (\%) = \frac{(A-B)}{A\times 100}\ \text{Equation} \quad (3)$$

AChE inhibitory assay

AChE inhibitory activity by the hydrolysates was assessed as described by Ellman et al. [33] For that, a *Tris*-HCl solution (0.05 M, pH 7.5) containing AChE from *Electrophorus electricus* at 1.25 U/mL was first prepared. The reaction medium was prepared in a 96-well plate and composed of 100 µL of potassium phosphate buffer (TFK, 100 mM, pH 7.5), 20 µL of water, 10 µL of solution containing the AChE enzyme and 20 µL of protein hydrolysate solution at concentrations which were defined by preliminary tests: 20, 30 and 40 mg/mL). The control sample was prepared without the addition of protein hydrolysate (20 µL of water).

First, the reaction medium was incubated at 25°C for 10 minutes. Then it was added 20 µL of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB, 2mM) and 20 µL of solution containing the substrate acetyl thiocholine (ASCh, 8 mM in Milli-Q water). Absorption readings were performed every minute for 4 minutes at a wavelength of 405 nm using a plate reader (Thermo-Plate Reader). The results were expressed as percentage of activity in relation to the control (100%). Samples were analyzed in quadruplicate.

Statistical Analysis

Proximal composition and amino profile characterization were evaluated by t-Student's test. The antioxidant capacity tests (DPPH, FRAP, ABTS, and TBARS) and acetylcholinesterase (AChE) inhibition were evaluated by t-Student's test regarding enzyme type and by ANOVA followed by the Tukey test regarding hydrolysate concentration. All results were analyzed in Matlab software (R2022b, Mathworks) with a significance level of 95%.

RESULTS AND DISCUSSION

Chicken Sternal Cartilage Proximate Composition

The chicken sternal cartilage was analyzed in relation to its proximate composition, and the results are presented in Figure 1 (A). The chicken sternal cartilage hydrolysis process catalyzed by the enzymes Alcalase and Flavourzyme resulted in significantly different degree of hydrolysis ($p < 0.05$), as presented in Figure 1 (B).

As can be seen, the protein was the major component (48.18%$_{wb}$, 58.95%$_{db}$) found in the chicken sternal cartilage, confirming that it is an important source for protein hydrolysates production. Akram and Zhang [34] also studied chicken sternal cartilage as a source of protein hydrolysates and found values of protein content between 70 and 80%$_{wb}$ after treating the cartilage with ultrasound. The moisture content found by the authors was 3.6-fold lower than the determination of the present study. Also, Shen et al. [35] identified a protein content of 73.35%$_{db}$ in chicken sternal cartilage before it was subjected to hot pressure by membrane combination separation technology that generated a liquified cartilage.

Lipids, and ashes represented a small portion of chicken sternal cartilage, and the results of Figure 1 (A) showed little variation compared to other studies [34–36].

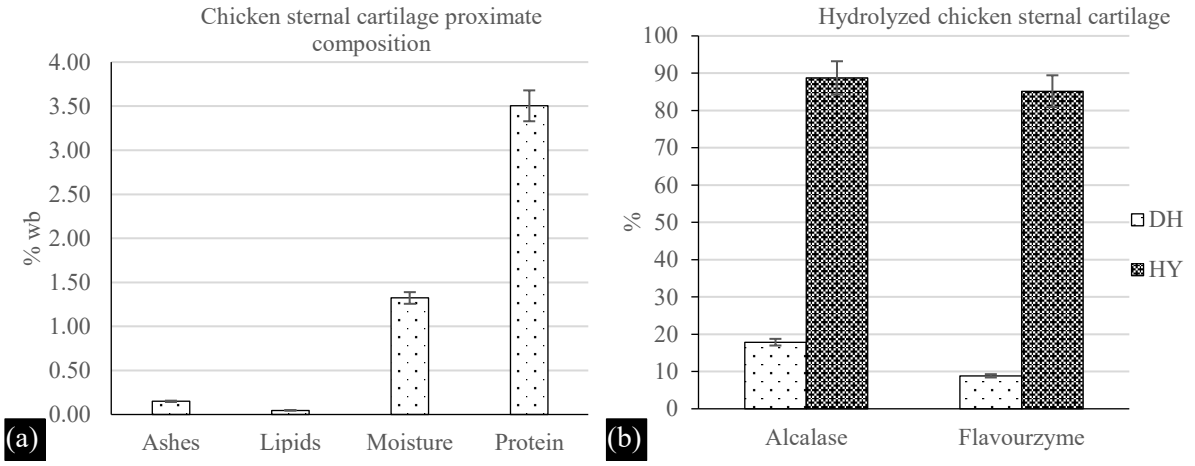
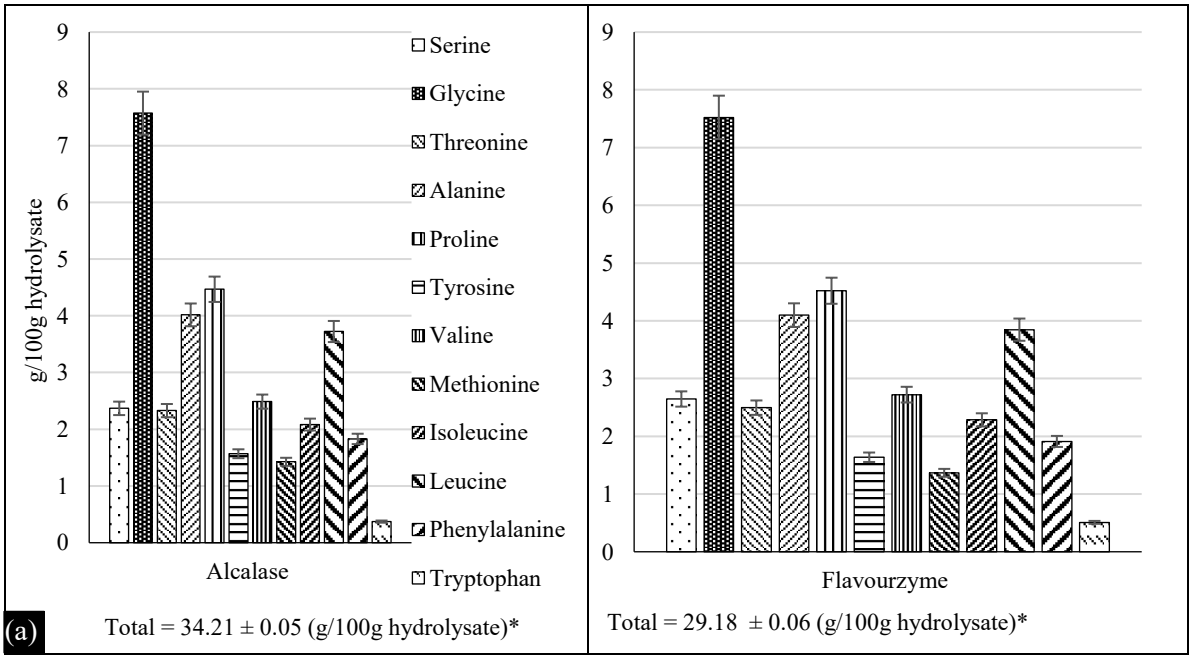


Figure 1. (A) Chicken Sternal Cartilage proximate composition (wet basis); (B) Degree of Hydrolysis (DH) and Yield (HY) determined after Alcalase or Flavouzyme enzymatic hydrolysis of chicken sternal cartilage. * Indicates significant difference by t-Student's test ($p < 0.05$).

Alcalase promoted a 2.2-fold higher capacity in hydrolyzing the Chicken sternal cartilage than Flavourzyme, leading the cleavage of protein in peptides and free amino acids [37,38]. Also, both treatments presented high recovery (<93%) of soluble protein. According to Ovissipour et al. [39] from an economic standpoint, enzymatic hydrolysis yield is a crucial outcome, thus results from Figure 1 (B) suggest that the use of both Alcalase and Flavourzyme may result in viable processes.

Hydrolysate's Amino Profile Characterization

The amino acid profile of the chicken sternal cartilage hydrolysates is presented in Figure 2. A total of 18 amino acids were identified for the two samples analyzed, 6 hydrophilic and 12 hydrophobic. Glycine and glutamic acid emerged as the predominant amino acids among those identified. Upon examination of Figures 2 (A) and (B), it becomes evident that Flavourzyme and Alcalase enzymes yielded hydrolysates with varying levels of amino acids. Notably, tyrosine and tryptophan, both classified as hydrophobic amino acids, along with histidine, a hydrophilic amino acid, exhibited statistical similarities ($p > 0.05$) across the treatments.



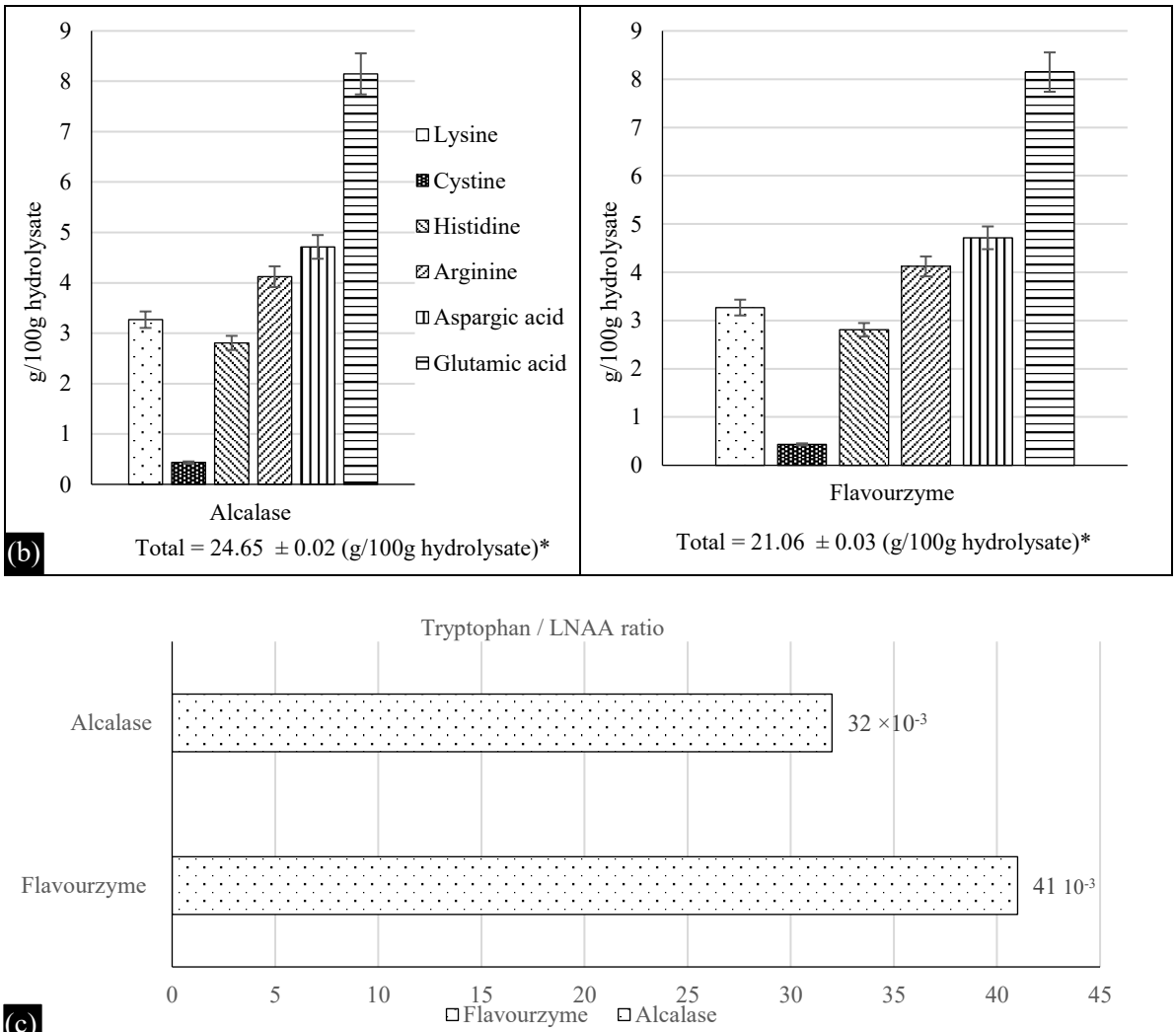


Figure 2. (A) Hydrophobic amino acid profile of chicken sternal cartilage hydrolysates; (B) Hydrophilic amino acid profile of chicken sternal cartilage hydrolysates; (C) Tryptophan/LNAA (Large Neutral Amino Acids: leucine, isoleucine, valine, phenylalanine and tyrosine) ratio. * Indicates significant difference by t-Student's test ($p < 0.05$).

The variation in amino acid composition of protein hydrolysates mainly depends on several factors such as raw material, enzyme source, and hydrolysis conditions [40]. Also, proteolysis not only leads to the release of additional amino acids compared to the raw material but can also cause the degradation or complexation of specific amino acids [41]. These may explain the difference found between the two enzymes.

While the hydrolysate generated with Flavourzyme exhibited a lower total amino acid content than that produced with Alcalase, its tryptophan/LNAA ratio was 1.28-fold higher ($p < 0.05$). Amino acids are crucial in regulating neurotransmitter availability within the central nervous system. For instance, the synthesis of brain serotonin relies heavily on the concentration of its precursor, tryptophan [42]. Tryptophan is the sole precursor of the mood-regulating neurotransmitter serotonin. Studies have shown that increased serotonin levels in the brain enhance mood and reduce emotional disturbances in healthy individuals, while decreased serotonin levels in the brain lead to depression and anxiety [43]. The biosynthesis of serotonin in the brain relies on the availability of tryptophan, which, in turn, depends on the ratio of other neutral amino acids of higher mass (or large neutral amino acids, LNAA), specifically leucine, isoleucine, valine, phenylalanine, and tyrosine. Therefore, consuming supplements with a relatively high ratio of tryptophan to LNAA may yield better post-consumption mood-related

outcomes than supplements containing only tryptophan, owing to the positive interactions among the constituents [44]. This effect occurs because tryptophan competes with other LNAA for transport across the blood-brain barrier (BBB); thus, increasing the tryptophan-to-LNAA ratio enhances the likelihood of tryptophan accessing BBB transporters [45].

Antioxidant Capacity and AChE Inhibition by Hydrolysates

Table 1 presents the results of the antioxidant capacity and AChE inhibition by the chicken sternal cartilage hydrolysates. Among the antioxidant assay results, the only statistically significant difference ($p < 0.05$) detected was for TBARS, where the hydrolysate produced with Flavourzyme had a 1.3-fold higher inhibition capacity. Although Flavourzyme has generated a hydrolysate with a lower overall amount of amino acids, the same level of tryptophan content was detected in the hydrolysate produced with Alcalase. Consequently, the higher tryptophan/LNAA (Large Neutral Amino Acids) ratio in the sample hydrolyzed with Flavourzyme positively influenced the TBARS reduction.

Thiobarbituric acid reacting substances (TBARS) are products formed as a result of free radical-induced lipid peroxidation in the human body. These substances are associated with oxidative stress markers, showing an imbalance between the number of free radicals and antioxidants in the body [46,47]. In a study by Medeiros et al.,[48[NO_PRINTED_FORM] 48], oxidative stress parameters including TBARS, 2′,7′-dichlorofluorescein oxidation assay (DCF), sulfhydryl content, and activities of superoxide dismutase (SOD) and catalase (CAT) were evaluated in zebrafish exposed to high levels (2 mM and 5 mM) of leucine, a component of LNNA. Results from brain tissue analysis after 24 hours of exposure revealed an increase in oxidative stress.

Additionally, Barschak et al. [48] assessed various oxidative stress parameters including TBARS, total antioxidant reactivity (TAR), and total antioxidant status (TAS) in the plasma of MSUD patients. Maple Syrup Urine Disease (MSUD) or Branched-Chain α-Keto Aciduria (BCKA) results in the accumulation of branched-chain amino acids (BCAA) such as leucine, isoleucine, and valine, all of which are LNNA components. The study found a significant increase in plasma TBARS measurements, indicating heightened lipid peroxidation, along with a decrease in plasma TAR, suggesting an impaired ability to effectively counteract damage associated with increased reactive species production.

Table 1. Antioxidant capacity (DPPH, FRAP, ABTS, and TBARS) and acetylcholinesterase (AChE) inhibition by the chicken sternal cartilage hydrolysates obtained with Alcalse and Flavourzyme.

Hydrolysis Enzyme		**Antioxidant Capacity**		
	µmol$_{ET}$/g$_{sample}$ *(µmol$_{ET}$/mL)			***TBARS$^{\#}$ % inhibition***
	DPPH$^{\#}$	*FRAP‟*	*ABTS‟*	
Alcalase	$56.00^{A} \pm 2$ *(1.67 ± 0.06)	$12.20^{A} \pm 0.4$ *(0.37 ± 0.01)	$30.91^{A} \pm 0.06$ *(0.927 ± 0.01)	$56.0^{B} \pm 2.0$
Flavourzyme	$53.00^{A} \pm 3$ *(1.59 ± 0.09)	$11.20^{A} \pm 0.3$ *(0.34 ± 0.01)	$31.18^{A} \pm 0.06$ *(0.935 ± 0.01)	$74.2^{A} \pm 0.7$
	AChE Inhibition Potential (%)			
	Hydrolysate Concentration (mg/mL)	20	30	40
Alcalase		$37^{c.A} \pm 2$	$46^{b.A} \pm 3$	$61^{a.A} \pm 3$
Flavourzyme		$28^{a.B} \pm 3$	$30^{a.B} \pm 3$	$32^{a.B} \pm 3$

**Results expressed as µmol$_{ET}$/mL of sample solution; $^{\#}$Samples evaluated at 20 mg/mL; ‟Samples evaluated at 30 mg/mL; ^{a,b,c} Different letters in the same row mean statistical difference ($p < 0.05$) by ANOVA followed by the Tukey test; ^{A,B} Different letters in the same column mean statical difference ($p < 0.05$) by t-Student test.*

Hence, the higher tryptophan/LNAA ratio observed in the hydrolysate produced by Flavourzyme may have contributed to the reduction of lipid peroxidation in the analyzed sample in the current study (Table 1).

Regarding AChE inhibition potential, the hydrolysate generated with Alcalase demonstrated results that were 1.86 times higher than those produced with Flavourzyme. Additionally, there was no statistically significant difference ($p > 0.05$) among the results for the three concentrations tested with Flavourzyme-derived hydrolysate.

The correlation between bioactive properties and protein hydrolysates composition has already been reported in the literature. [49–51] The fact that since low molecular weight peptides have stronger antioxidant activity was demonstrated by the DPPH method in which lower average molecular weight samples revealed a stronger reducing power [52].

In the work of Moreira et al. [25] the authors produced fish protein hydrolysates obtained by Alcalase enzymatic hydrolysis and determined by molecular docking simulation that L-arginine was the amino acid most likely to bind to AChE, also that the sample with higher content of amino acids was the one with the highest AChE inhibitory potential (26.19% at 30 mg/mL and 40.45% at 40 mg/mL). The same trend was observed in the present work in relation to the sample with the highest amino acid content.

In a study carried out by Jongsriwattanaporn et al. [53] a hydrolysate was produced from chicken breast broth using serine endopeptidase (EC 3.4.21.62) from *Bacillus licheniformis*. The authors assessed the hydrolysate's AChE inhibitory potential at 5 mg/mL, revealing a 50.64% inhibition, which exceeded the inhibition observed in our current study. Despite using a significantly higher hydrolysate concentration (40 mg/mL), the maximum inhibition achieved in the present work was 60.65%. According to Amouheydari, Ehsani and Javadi, [54] the amino acid terminals of hydrolysates are directly linked to their biological activities and act differently depending on the enzyme used for hydrolysis, among other process conditions.

The objective of this research was to evaluate chicken sternal cartilage's potential as a protein hydrolysate source, as well as to elucidate hydrolysate's bioactive properties, namely, antioxidant and AChE inhibitory potential. The research has illustrated that chicken sternal cartilage emerges as a promising protein source for hydrolysate production. This is owed to its substantial protein content, alongside achieving commendable degrees of hydrolysis and yields with both applied enzymes. Specifically, Alcalase proved notable for generating a chicken sternal cartilage hydrolysate rich in total amino acids, thereby enhancing its potential for AChE inhibition. Conversely, Flavourzyme exhibited efficacy in producing a hydrolysate characterized by a higher tryptophan/LNNA ratio, likely contributing to enhanced TBARS inhibition.

CONCLUSION

The potential of chicken sternal cartilage as a source of protein hydrolysates was evaluated, elucidating the bioactive properties of these hydrolysates, specifically their antioxidant and acetylcholinesterase (AChE) inhibitory potentials. The main results revealed that the hydrolysate produced with Alcalase exhibited a high total amino acid content, which was associated with a notable AChE inhibitory potential. In contrast, the hydrolysate produced with Flavourzyme showed a higher tryptophan/large neutral amino acids (LNAA) ratio, likely contributing to the reduction of lipid peroxidation, as evidenced by its enhanced TBARS inhibition. The findings contribute valuable insights to the field of food engineering, particularly in the development of functional food ingredients with health-promoting properties. The study successfully demonstrated that chicken sternal cartilage is a valuable protein source for hydrolysate production, owing to its high protein content and significant degrees of hydrolysis and yields achieved with both Alcalase and Flavourzyme enzymes.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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