




Article

Sequential Hydrolysis of Chicken Feathers Composed of Ultrasound and Enzymatic Steps: An Enhanced Protein Source with Bioactive Peptides

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Abstract: Chicken feather is a massive by-product. Its incorrect disposal can lead to serious environmental impacts. However, chicken feather is a promising low-cost keratin source. Keratin products have a wide application in the food and pharmaceutical industry. Mostly, chicken feathers are hydrolyzed by hydrothermal processes, and then applied into animal feed formulations. Despite the low cost, the hydrothermal hydrolysis leads to uncontrolled and low hydrolysis yield. Therefore, the aim of this work was to develop and optimize a sequential strategy of chicken feathers hydrolysis composed of ultrasound and enzymatic hydrolysis (savinase[®]) steps. In the first research step an experimental design was built and the optimum hydrolysis condition was obtained at 50 °C and 12.5% (enzyme/chicken feather), using three integrated reactors containing enzyme/substrate and sodium disulfite. Then, the ultrasound probe was added in the experimental apparatus in order to investigate the enzymatic hydrolysis assisted by ultrasound treatment. The enzymatic hydrolysis assisted by ultrasound treatment led to high concentrations of peptides, including a dipeptide (245.1868 *m/z*). Thus, the sequential hydrolysis strategy composed by two green technologies proposed in this study, enhanced the degree of hydrolysis of chicken feathers, producing bioactive peptides that can be used as ingredients in food products and other sectors.

Keywords: chicken feathers; hydrolysate; peptides



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1. Introduction

The incorrect disposal of keratinous residues can lead to serious environmental impacts, since >60% of keratinous residues are disposed in landfills, dumpsites, and incinerators (very slow degradation rate, landscape deterioration, pollution, transmission of disease pathogens, among others) [1].

These residues are, inherently, generated at global scale, for instance ≈1.5 Mt of sheep wool, ≈9.5 Mt of poultry feathers, ≈5 Mt of human hairs, and ≈1 Mt of horns [1]. Thus, poultry feathers are the most abundant keratinous residues (≈40%), of which China, India, the USA, and Brazil are the most representative countries.

According to the USDA, in 2021, the total number of broilers produced was 9.13 billion. Only in Brazil, has chicken production increased ≈4%, reaching a record of 14.4 Mt. This represents 1.17 Mt of chicken feathers (CFs) as a by-product [2].

CFs are composed, essentially, of proteins (≥75), in which ≈90% it is keratin [3]. Keratin, a crystalline protein, can be classified into two general classes based on amino acid composition, distribution, and function: type I (the acidic keratins) and type II (the basic keratins). The acidic keratins subdivide into acidic hard and acidic soft keratins [1]. The average molecular weight of feather proteins is ≈10,000 g·mol⁻¹ (10 kDa) [4].

Thus, keratinous residues have remarkable potential as sources of proteins, peptides, amino acids, energy sources, raw materials, and feedstock for a wide range of industrial and commercial high value-added applications [1], for instance gels, films, spheres, nano, and microparticles [5,6], animal feed (extruded product composed of cornmeal and feather hydrolysate) [7,8], agriculture, and human feed and biofuel production [9]. The antioxidants bioactive peptides can be applied in food formulation to prevent or inhibit oxidation in food products. In addition, antibacterial and antifungal peptides have been part of a large group of antimicrobial peptides [10,11], antityrosinase, and inhibitors of angiotensin-converting enzyme (ACE) [12], as well as pharmaceuticals [13].

Thus, it is clear that the valorization of keratinous residues is correlated to hydrolysis steps. Usually, the feather hydrolysis method consists of a hydrothermal process at high pressures (from 300 to 350 kPa) and high temperatures (from 133 to 150 °C). However, the hydrothermal treatment is inefficient and time-consuming. In addition, thermal treatments induce racemization and amino acid oxidation, which leads to poor nutritional quality [6,14]. In this sense, environmentally friendly alternatives have been investigated, including enzymatic hydrolysis. In addition, the ultrasound treatment, which is also an eco-friendly and non-thermal technology, can be integrated into the enzymatic hydrolysis, since cavitation can change the protein hydration, molecular size, hydrophobicity, and conformation [15,16]. Thus, the ultrasound treatment, prior to enzymatic hydrolysis can increase exposure of protein groups (higher superficial area due to the unfolding of protein structure) and also the partial hydrolysis of proteins. These effects mostly favor the activity of endoproteases, such as savinase (savinase®). Therefore, the aim of this work was to develop and optimize a sequential strategy of chicken feathers hydrolysis composed of ultrasound and enzymatic hydrolysis (savinase®) steps.

2. Materials and Methods

2.1. Raw Materials and Reagents

CFs were kindly donated by Frangos Morgana poultry processing company (Palhoça-SC/Brazil). Savinase® (16L TYPE EX, EC.3.4.21.62, lot A-68546-Novozymes) was donated by Elberbio Research and Development Ltd. (Florianópolis-SC/Brazil). Detergent was purchased from a local supplier. Sodium sulfite (Na_2SO_3), sodium hydroxide (NaOH), and formaldehyde 37% (CH_2O) were purchased from Neon (Suzano-SP/Brazil). The other reagents used in the research were purchased from Sigma Aldrich (Jurubatuba-SP/Brazil).

2.2. Chicken Feather Pre-Treatment

In order to remove lipids, CFs were washed (tap water), and then immersed in an aqueous solution containing $1 \text{ g} \cdot \text{L}^{-1}$ household detergent (linear alkyl benzene sodium sulfonate, among others) for 30 min at 45 °C. Then, CFs were rinsed three times with tap water at 45 °C, and two times with distilled water [17]. CFs were oven-dried at 45 °C for 12 h (SP-400, SPLabor, Presidente Prudente-SP/Brazil) and processed in a knife mill (TE-648, Tecnal, Piracicaba-SP/Brazil). The ground CFs were packaged in a hermetically sealed low-density polyethylene plastic bag and stored at $-16 \text{ }^\circ\text{C}$.

2.3. Centesimal Composition of Feathers

Proximate composition analyses of raw feathers were performed according to Association of Official Analytical Chemists—AOAC [18] moisture, ash, total proteins, and lipids (methods 950.46, 920.153, 928.08, and 920.39, respectively).

2.4. Alkaline Dissolution of Ground Chicken Feathers

Preliminary experiments with CF dissolution were performed in an integrated system composed of three reactors (300 mL) and thermostatic bath (0214M2, Quimis, Diadema-SP/Brazil) under a magnetic stirrer (1000 rpm) (Figure 1). Ground CFs ranging from 1.32 to 4.68% (m/v) and sodium sulfite (antioxidant) from 0.05 to 0.11 M were added into reactors,

with 100 mL of 0.08 M NaOH solution (pH 12.9) and then, into the mixture for 12 h. The degree of hydrolysis was measured every two hours for 12 h [19,20].

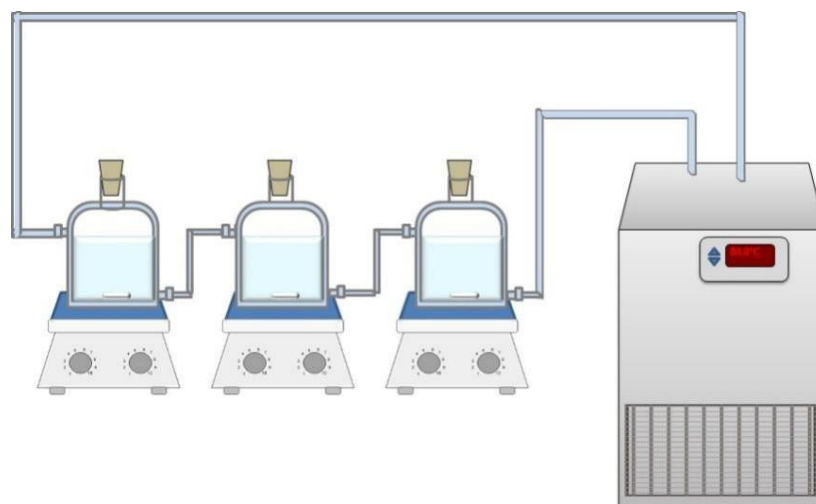


Figure 1. Integrated system: three reactors and thermostatic bath.

2.5. Optimization of Enzymatic Hydrolysis

After the alkaline dissolution of ground chicken feathers, an enzymatic hydrolysis was carried out in the same integrated system composed of three reactors (300 mL) and a thermostatic bath (0214M2, Quimis, Diadema-SP/Brazil SP/Brazil) under magnetic stirring (60 rpm) (Figure 1). The enzyme manufacturer's recommendations (Novozymes) detail the working temperature range from 55 to 75 °C and pH range between 7 and 11. The preliminary experiments with ground chicken feathers achieved a higher hydrolysis yield at 50 °C and pH 10.6. Thus, pH was adjusted to 10.6 with HCl (3N). Alkaline dissolution results were considered for CF concentration (4%).

Then the savinase[®] (16L TYPE EX, EC.3.4.21.62, lot A-68546-Novozymes) was evaluated at different enzyme/substrate (E/S) ratios ranging from 1.9 to 23.1, according to the design of experiments 2² (Table 2)—Independent variables: E/S ratios and temperature; and degree of hydrolysis (GH) as dependent variable.

The enzymatic hydrolysis was monitored by the GH every two hours for 10 h according to Sorensen [18]. The protease savinase[®] was thermally inactivated (80 °C for 10 min).

2.6. Sequential Hydrolysis Composed of Ultrasound and Enzymatic Steps

It was evaluated for the ultrasound effect on chicken feather hydrolysis, prior to the enzymatic step (optimal condition—previously identified). Thus, after the alkaline dissolution of ground chicken feathers, 50 mL of solution were transferred to a plastic beaker and cooled in an ice bath. The optimal sonication condition was determined by design of experiments 2² (Table 5) using a probe sonicator (QR500, Eco-sonics, Indaiatuba-SP/Brazil) at 20 kHz and ultrasonic power between 200 and 442 W, for 5 to 23.1 min. The 4 mm diameter titanium micro-point probe was placed centrally, at 0.5 cm depth into the solution.

2.7. Microfiltration of Amino Acids and Peptides

In order to purify amino acids and peptides, the CF hydrolysates were neutralized to pH 7.5 with HCl (5N), and then microfiltrated at 28 °C and 2 bar by using a stainless steel cell under magnetic stirring and 0.2 µm polyvinylidene difluoride membrane (MV020, Microdyn Nadir, São Paulo-SP/Brazil) with area of $1.02 \times 10^{-3} \text{ m}^2$, previously hydrated with distilled water for 20 min.

2.8. Analytical Methods

2.8.1. Degree of Hydrolysis

The degree of hydrolysis was measured according to the method of Sorensen [18]. Firstly, the 50 mL of formaldehyde–phenolphthalein solution was prepared, in which 1 mL of 0.05% phenolphthalein (50% ethanol solvent) was added to formaldehyde (CH₂O 37%). The pH was adjusted to 8.5 with Ba(OH)₂ 0.2 N and HCL 0.2 N. Then, 10 mL of formaldehyde–phenolphthalein reaction solution was mixed with 10 mL of feather hydrolysates also adjusted to pH 8.5. Blank samples were prepared with an equivalent volume of distilled water and neutralized formalin solution. The α -amino nitrogen was measured by Equation (1):

$$\alpha\text{-amino nitrogen} = [(V_{\text{Ba(OH)}_2} - V_{\text{control}}) \times f \times N_{\text{Ba(OH)}_2} \cdot 0.014 \cdot \times 100] / \text{sample weight in aliquot}, \quad (1)$$

The degree of hydrolysis was measured by Equation (2):

$$\text{Degree of hydrolysis (\%)} = (\alpha\text{-amino nitrogen} / \text{total nitrogen}) \times 100 \quad (2)$$

Total nitrogen was determined by Kjeldahl method, 928.08 [18].

2.8.2. Ultrasound Effect on Particle Size Distribution of Dissolved Feathers

The ultrasound (50 mL; 450 W; 12.5 min) effect on the particle size was analyzed by Dispersion Analyser Lumisizer[®] (LS 611, L.U.M. GmbH, Jiangsu province/China) 25 °C, 4000 rpm.

2.8.3. Peptide Profile

The keratin hydrolysates peptide profile (molecular mass, distribution of peptides: di-, tri- and oligopeptides) was determined by hydrophobic interaction liquid chromatography coupled to mass ionization by nebulization and qTOF analyzer (HILIC-ESI-qTOF-MS), detailed below:

We used an ACQUITY UPLC system class H (Waters, Milford, MA, USA) equipped with a photodiode array (PDA) detector, sample manager, and a quaternary solvent manager. A HILIC column (100 mm, 2.1 mm, particle size 2.7 μm (Supelco, Sigma-aldrich, Jurubatuba-SP/Brazil) was used for the separation. The column and the sample tray were maintained at temperatures of 40 °C and 20 °C, respectively. The sample (3 μL) was injected and separated with a gradient condition at a flow rate of 0.4 mL/min. Solvent A was composed of acetonitrile (ACN) and H₂O (90:10) and 0.1% of formic acid (*v/v*); B was composed of H₂O and ACN (90:10). The elution was performed using the following gradient condition: 0–2 min, 95% of A; 2–10 min, 55% of A; 10–15 min, 5% of A; 15–20 min, 95% of A. A Xevo G2-SQToF (Waters, Wexford/Ireland) bearing an electrospray ionization (ESI) probe operating in positive and negative ionization modes coupled to the UPLC device was used to detect the chemical components of each sample. Nebulizer gas: nitrogen; cone gas flow: 100 L/h; desolvation gas flow: 900 L/h; sampling cone 40 V; source offset 80 V; collision gas: argon; lockspray reference sample was leucine enkephalin with reference masses at *m/z* 554.2615 (ESI[−]). The desolvation and the ionization source were maintained during the analyses at 250 °C and 90 °C, respectively, while the capillary voltage was 3 kV. A range of 25 to 35 eV was used as the collision energy. Data were acquired in a range of 100–1500 Da, at a scan time of 1.0 s over 20 min and were processed with MassLynx V4.1 (Waters, Wexford/Ireland). Molecular formulas were determined by calculation using MassLynx's elemental composition tool. The molecular formula was restricted by a tolerance of 5 ppm between the calculated and the measured mass values.

2.8.4. Biological Properties of Chicken Feathers Hydrolysate

Digestibility

The digestibility of CF hydrolysates *in vitro* was carried out according to Tiwary and Gupta [5]. Briefly, hydrolysates were diluted into HCl 0.1 N, then pepsin was also added (2 mg/mL). It was incubated at 37 °C for 2 h. After the acid digestion, it was alkalinized

with NaOH 2 N, then trypsin was added (2 mg/mL) and incubated at 37 °C for 16 h. All samples were centrifuged 6000× g and the supernatants measured at 660 nm (bovine serum albumin curve).

Antioxidant Activity

Antioxidant activities of hydrolysates were measured by capturing free radical methods (DDPH and ABTS) [21,22].

2.9. Statistical Analyses

Statistical analyses were carried out using design of experiments (DOE), according to Rodrigues and Lemma [23], at 10% significance level by using Statistica 7.0 software.

3. Results

3.1. Proximate Composition of Feathers

Proximate composition of feathers is presented in Table 1, where it can be observed that the feathers are composed mainly of proteins, around 84%. The centesimal composition of CFs (Table 1) was similar to that reported by Fakhfakh et al. [24] who obtained $85.31\% \pm 0.43$ of protein. It is noteworthy that CF protein content is remarkably high $\geq 75\%$, of which $\approx 90\%$ out of these proteins are keratin [25].

Table 1. Centesimal composition of feathers.

	Average Value (%)
Proteins	84.28 ± 3.91
Lipids	8.08 ± 0.39
Moisture	7.98 ± 0.24
Ash	0.42 ± 0.02

3.2. Alkaline Dissolution of Ground Chicken Feathers

The results of preliminary dissolution experiments showed that the highest concentration of ground chicken feathers (4% *m/v*) led to higher degrees of hydrolysis. On the other hand, sodium sulfite tended to be more efficient at lower concentrations (0.06 M). The disulfide bonds, widely found in chicken feather proteins, can be cleaved by sodium sulfite [19]. Thus, it is very likely that sodium sulfite affects savinase[®] activity, which aligns with research published by Adler et al. [26], who investigated the hydrolysis of CFs by thermal treatment (2 bar, 121 °C, 15 min) with sodium sulfite and/or sodium hydroxide. The authors concluded that sodium hydroxide, sodium sulfite, and thermal treatment improved the hydrolysis of CFs. It should be noted that these molecules have complex interactions and different effects, which can, in principle, counteract each other, such as changed bonding, increased/decreased crosslinking, changes in polarity, and network disturbance by additives. In addition, as expected, high temperatures (80 to 97 °C) also produced higher degrees of hydrolysis. However, thermal treatments induce racemization and amino acid oxidation—poor nutritional quality [2,14,27–30].

Therefore, the following condition of alkaline dissolution was chosen for the enzymatic hydrolysis (4% substrate, 70 °C, and 0.06 M sodium sulfite for 8 h).

3.3. Optimization of Enzymatic Hydrolysis of Chicken Feathers

Design of experiments 2² was used to investigate the optimal condition of enzymatic hydrolysis (Table 2).

Table 2. Design of experiments 2² enzymatic hydrolysis (Savinase®) of ground chicken feathers.

Experiments	E/S * (%)	T (°C)	Degree of Hydrolysis (%)				
			2 h	4 h	6 h	8 h	10 h
1	5 (−1)	45 (−1)	3.2	5.3	5.3	5.3	5.3
2	5 (−1)	55 (1)	5.3	5.3	6.3	6.3	6.3
3	20 (1)	45 (−1)	5.3	5.3	5.3	5.3	6.3
4	20 (1)	55 (1)	5.3	5.3	5.3	6.3	7.4
5	1.9 (−1.414)	50(0)	5.3	5.3	5.3	6.3	6.3
6	23.1 (1.414)	50 (0)	6.3	6.3	8.4	8.4	8.4
7	12.5 (0)	43 (−1.414)	5.3	6.3	6.3	6.3	6.3
8	12.5 (0)	58 (1.414)	4.2	4.2	4.2	4.2	4.2
9	12.5 (0)	50.0 (0)	7.4	7.4	8.4	8.4	8.4
10	12.5 (0)	50.0 (0)	5.3	7.4	8.4	8.4	8.4
11	12.5 (0)	50.0 (0)	5.3	6.3	7.4	7.4	7.4

* E/S enzyme/substrate concentration ratios = 5%, equals to 0.2% (*w/w*) savinase/total solution. Alkaline dissolution 4% (*m/v*), 70 °C, and 0.06 M Na₂SO₃ for 8 h.

It is worth noting that the degree of hydrolysis was measured every two hours. Thus, five dependent variables were obtained. The regression coefficients and analysis of variance (ANOVA) for response variables are described in Table 3. Regarding the 10 h statistical model, it presented significance and predictability. In addition, it showed the highest R².

Table 3. Enzymatic hydrolysis—Regression coefficients and analysis of variance (ANOVA) for response variables.

Coefficients	Degree of Hydrolysis (%)				
	2 h	4 h	6 h	8 h	10 h
β_0	6.00 *	6.40 *	8.07 *	7.61 *	7.74 *
Linear					
β_1	0.44	0.18	0.42	0.37	0.63 *
β_2	0.07	−0.37	−0.74 *	−0.48	−0.35
Quadratic					
β_{11}	−0.23	−0.68	−0.25	−0.12	−0.11
β_{22}	−0.75	−0.76 *	−1.54 *	−1.40 *	−1.31 *
Interactions					
β_{12}	−0.53	0.00	−0.25	0.00	0.03
R ²	0.53	0.36	0.61	0.60	0.74
F _{calculated}	-	5.10	6.20	13.72	11.45
F _{tabulated}	-	3.36	3.11	3.36	3.11
F _{lack of fit}	-	1.90	4.12	3.08	2.05
F _{tabulated*}	-	9.35	3.46	9.35	9.33
		SM and PM	SM	SM and PM	SM and PM

* It indicates significance at 90% confidence interval and pure error. ₁: enzyme/substrate ratio, ₂: temperature. F_{calculated} > F_{tabulated}: significant model (SM). F_{lack of fit} < F_{tabulated*}: predictive model (PM).

Hence, based on statistical analysis (significance, predictability, and R²) the response surface was plotted—10 h (Figure 2). Then, a second order coded model was elaborated by using the Statistica 7.0 software, Equation (3):

$$\text{Degree of hydrolysis} = 7.7 + 0.6 \times [\text{enzyme/substrate ratio}] - 1.3 \times [\text{Temperature}]^2 \quad (3)$$

Clearly, the optimal condition of enzymatic hydrolysis was reached at center points: 12.5% enzyme/substrate ratio and 50 °C, and a very low standard deviation was observed (*s* = 0.58%). The Kinect analyses showed that at 6 h, the degree of hydrolysis reached a stationary hydrolysis. Hence, the optimal condition of enzymatic hydrolysis can be carried out faster, for 6 h instead of 10 h.

According to Nyo and Nguyen [31] and Uluko et al. [32], a hydrolysis degree around 8% is quite desirable, since higher hydrolyzation can produce free amino acids instead of peptides, for instance 14.4% salmon by-products [33]; 5.07% for milk protein concentrate [32]; 12.5% for peanut protein [31]. Therefore, the optimal condition of enzymatic hydrolysis is related to center points (design of experiments), which leads to a suitable hydrolysis degree ($\approx 8\%$) after 6 h.

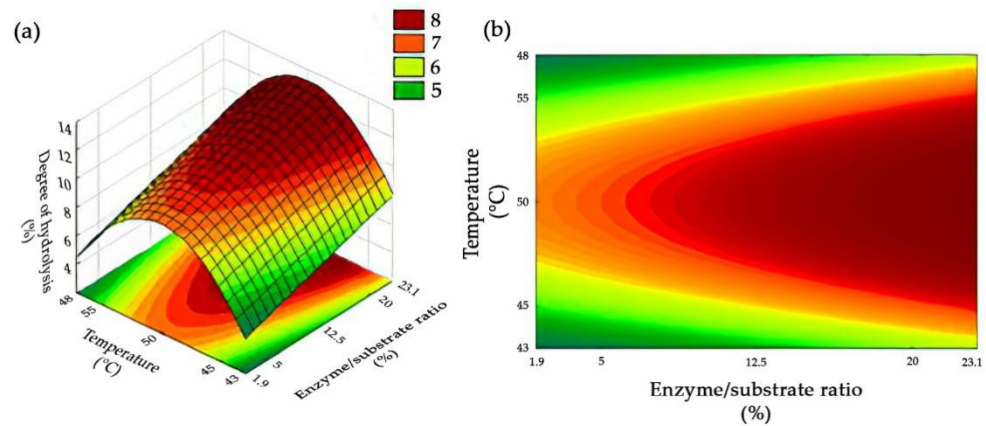


Figure 2. Response surface of enzymatic hydrolysis at 10 h. (a) Surface plot; (b) Counter plot.

3.4. Sequential Hydrolysis Composed of Ultrasound and Enzymatic Steps

In order to enhance the hydrolysis of CFs, a sequential hydrolysis of CFs composed by ultrasound and enzymatic steps was performed. The application of ultrasound (cavitation) on proteins, such as keratin, affects their hydration, molecular size, hydrophobicity, and conformation [15,16]. Thus, the ultrasound treatment, prior to enzymatic hydrolysis, can both increase exposure of protein groups and partially hydrolyze proteins. Thus, obviously, these effects favor the action of the enzyme. These effects are particularly desirable, since savinase (savinase[®]) is an endoprotease, that is, a slight exposition of keratin can significantly improve enzymatic activity. In this sense, the analysis of particle size (Table 4) indicated that ultrasound treatment reduced the particle size. The control samples showed an average particle size of 195 nm, whereas the sample treated with ultrasound presented lower average particle size (≈ 155 nm). Thus, the ultrasound treatment hydrolyzed partially, and/or destabilized protein aggregates, and/or changed the protein conformation.

Table 4. Particle size of chicken feathers, control, and treated samples with ultrasound.

	Mean *	10% ≤	16% ≤	50% ≤	84% ≤	90% ≤	Smallest	Largest
Control	194.9	163.3	167.1	193.7	323.0	404.2	150.7	911.8
Ultrasound	155.1	88.9	99.0	172.2	151.8	387.1	63.7	542.7

* (nm).

Thus, very likely, the sequential hydrolysis of chicken feathers using ultrasound followed by enzymatic hydrolysis can be applied to reach a higher degree of hydrolysis associated to the peptides production. Then, a design of experiments 2^2 (Table 5) was used to evaluate the optimal condition of sequential hydrolysis of chicken feathers using ultrasound and enzymatic hydrolysis.

It is worth noting that the degree of hydrolysis was measured every two hours. Thus, four dependent variables were obtained. The regression coefficients and analysis of variance (ANOVA) for response variables are described in Table 6, in which all dependent variables were significant and predictive models. Nevertheless, the 8 h statistical model showed the highest R^2 .

Table 5. Design of experiments 2² sequential hydrolysis composed of ultrasound and enzymatic steps.

Assay	Power (W)	Time (min)	Degree of Hydrolysis (%)			
			2 h	4 h	6 h	8 h
1	200.0 (−1)	5.0 (−1)	4.8	6.0	6.0	6.0
2	200.0 (−1)	20.0 (1)	7.2	7.2	7.2	7.2
3	400.0 (1)	5.0 (−1)	6.0	7.2	6.0	7.2
4	400.0 (1)	20.0 (1)	9.6	9.6	10.8	10.8
5	160.0 (−1.41)	12.5 (0)	7.2	7.2	7.2	7.2
6	442.0 (1.41)	12.5 (0)	9.6	9.6	10.8	10.8
7	300.0 (0)	1.9 (−1.41)	4.8	4.8	4.8	4.8
8	300.0 (0)	23.1 (1.41)	9.6	10.8	9.6	9.6
9	300.0 (0)	12.5 (0)	6.0	6.0	7.2	7.2
10	300.0 (0)	12.5 (0)	6.0	7.2	8.4	8.4
11	300.0 (0)	12.5 (0)	7.2	7.2	7.2	7.2

Table 6. Sequential hydrolysis composed of ultrasound and enzymatic steps—regression coefficients and analysis of variance (ANOVA) for response variable.

Coefficients	Degree of Hydrolysis (%)			
	2 h	4 h	6 h	8 h
β_0	7.09 *	7.53 *	7.75 *	7.85 *
Linear				
β_1	0.87 *	0.87 *	1.09 *	1.24 *
β_2	1.60 *	1.51 *	1.60 *	1.45 *
Quadratic				
β_{11}	0.78	0.65	0.55	0.63
β_{22}	0.17	0.35	−0.35	−0.28
Interactions				
β_{12}	0.30	0.30	0.9	0.60
R ²	0.81	0.76	0.78	0.81
F _{calculated}	16.51	12.87	13.89	17.60
F _{tabulated}	3.11	3.11	3.11	3.11
F _{lack of fit}	1.90	2.29	2.65	1.96
F _{tabulated*}	9.33	9.33	9.33	9.33
	SM and PM	SM and PM	SM and PM	SM and PM

* It indicates significance at 90% confidence interval and pure error. ₁: power (ultrasound), ₂: treatment time. F_{calculated} > F_{tabulated}: significant model (SM). F_{lack of fit} < F_{tabulated*}: predictive model (PM).

Hence, based on statistical analysis (significance, predictability and R²) the response surface was plotted—8 h (Figure 3). Then, a second order coded model was elaborated by using the Statistica 7.0 software Equation (4):

$$\text{Degree of hydrolysis} = 7.8 + 1.2 \times [\text{ultrasound treatment power}] + 1.5 \times [\text{ultrasound treatment time}] \quad (4)$$

Therefore, the sequential hydrolysis with ultrasound and enzyme reached a higher degree of hydrolysis (when compared to enzymatic hydrolysis without ultrasound). The eco-friendly ultrasound treatment should be integrated to the enzymatic hydrolysis of chicken feathers, since a higher degree of hydrolysis can be reached.

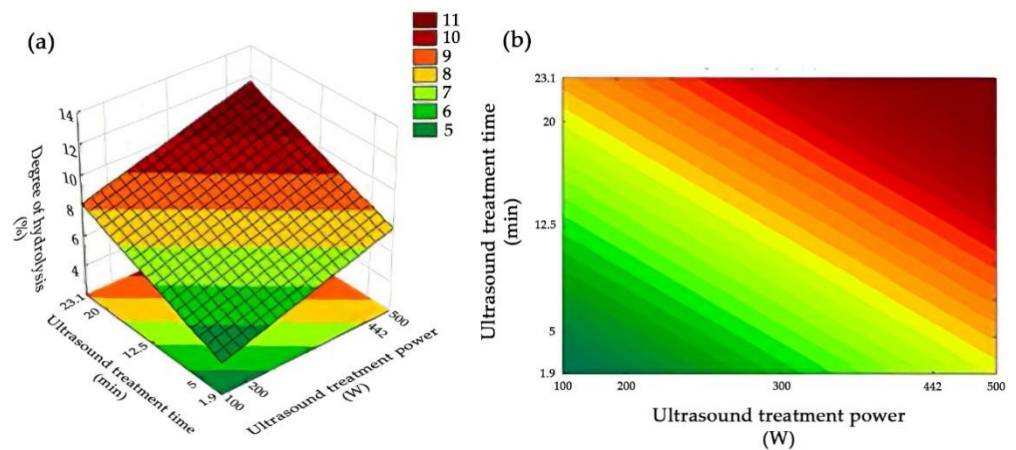


Figure 3. Response surface of sequential hydrolysis composed of ultrasound and enzymatic steps at 8 h. (a) Surface plot; (b) Counter plot.

3.5. Hydrolyzate Microfiltration

For hydrolyzate microfiltration on 0.2 μm membranes, a total flow of $\approx 2.93 \text{ L}\cdot\text{h}\cdot\text{m}^{-2}$ was obtained. Figure 4 shows the enzymatic hydrolyzate before and after microfiltration. The hydrolyzate was composed of ultrasound and enzymatic steps and had the same physical appearance. This operation was successful, obtaining purified hydrolyzates that were used in the analyses, including the peptide profile.

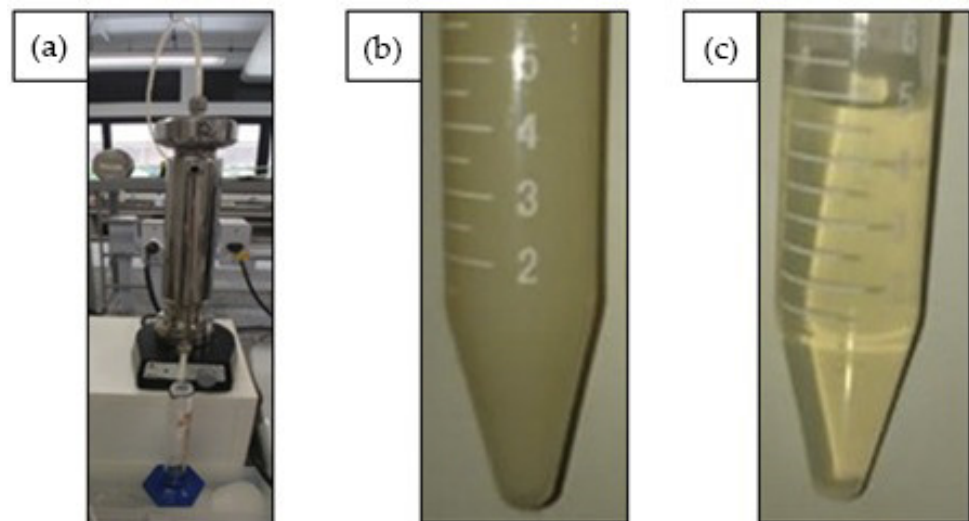


Figure 4. Hydrolyzates microfiltration. (a) Microfiltration equipment. (b) Feed: Chicken feather keratin hydrolyzate. (c) Permeate: Chicken feather keratin hydrolyzate.

3.6. Peptide Profile

Peptides are a diverse group of oligomeric structures, composed of short amino acid sequences, usually 2–20 residues. Peptides have remarkable biological properties, such as hormonal regulation, redox homeostasis, neuronal signal, cell signaling, transduction, growth, and immune response [34,35].

In mass spectra (Figures 5 and 6), the peaks with good resolution were selected, seven main ones for enzymatic hydrolyzate and six for hydrolyzate composed of ultrasound and enzymatic steps.

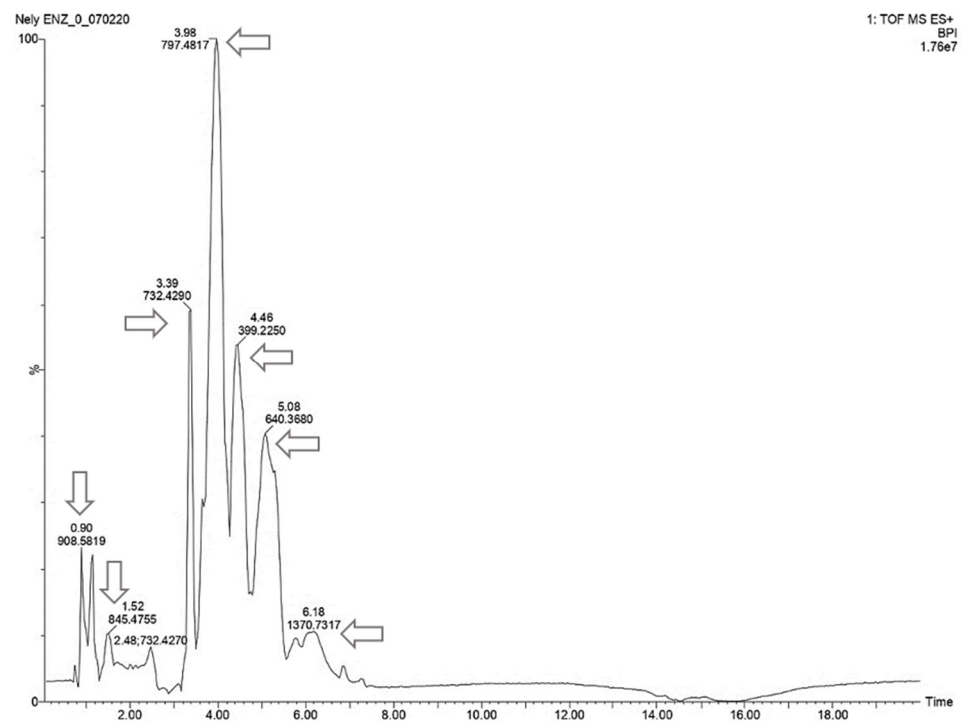


Figure 5. Mass spectrum (HILIC-ESI-QTOF-MS) of chicken feather keratin enzymatic hydrolyzate.

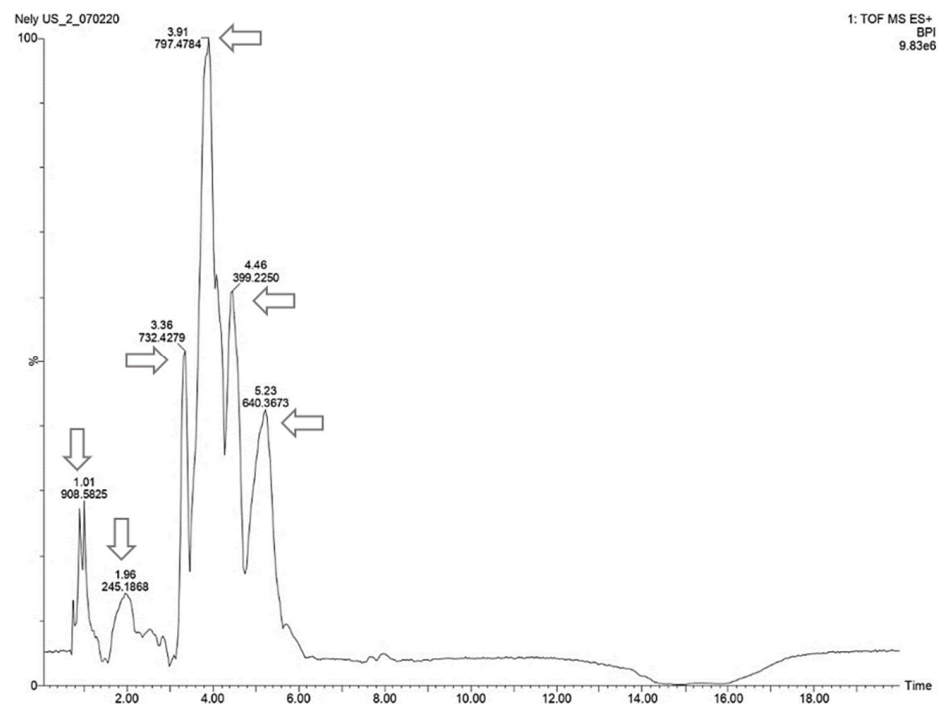


Figure 6. Mass spectrum (HILIC-ESI-QTOF-MS) of chicken feather keratin hydrolyzate composed of ultrasound and enzymatic steps.

Table 7 presents the peptide sizes that were obtained from chicken feather hydrolyzates by enzymatic hydrolysis and sequential hydrolysis composed of ultrasound and enzymatic steps. It is worth noting that the analytical method HILIC-ESI-qTOF-MS is quite sensitive to peptides composed of up to 20 amino acids [36].

Table 7. Peptide profile of chicken feather hydrolyzates.

Hydrolyzate	Measured (<i>m/z</i>)	Time (min)	Peptide Size
Enzymatic hydrolysis	399.2250	4.46	tripeptide
	640.3880	5.08	oligopeptide
	732.4290	3.39	oligopeptide
	797.4817	3.98	oligopeptide
	845.4755	1.52	oligopeptide
	908.5819	0.90	oligopeptide
	1370.7317	6.18	oligopeptide
Sequential hydrolysis composed of ultrasound and enzymatic steps	245.1868	1.96	dipeptide
	399.2250	4.46	tripeptide
	640.3673	5.23	oligopeptide
	732.4279	3.36	oligopeptide
	797.4784	3.91	oligopeptide
	908.5825	1.01	oligopeptide

Both hydrolyzates showed a similar peptide profile, however, the sequential hydrolysis composed of ultrasound and enzymatic steps showed higher intensity, which is related to the concentration of peptides. In addition, the sequential strategy produced the smallest peptide (dipeptide). Thus, it was proved that ultrasound-treatment affected the enzymatic hydrolysis. Further investigation should be carried out on the identification of amino acid sequences, purification strategies, mainly membrane-based technology, and application of these peptides.

3.7. Biological Properties of Chicken Feathers Hydrolyzate

CFs have low digestibility (9.6–15.6%) [5,26]. When compared to the enzymatic hydrolysis, the sequential hydrolysis composed of ultrasound and enzymatic steps showed higher digestibility, ≈ 80 and 100%, respectively (Table 8). These results are higher than chicken feather hydrolyzates that are commercially available $\approx 56\%$ and closer to feather protein hydrolyzate (86%) produced by Kshetri et al. [26,37], considering in vitro digestibility. It is worth noting that further experiments can be complementary to these in vitro protein digestibility trends, such as an approach with digestive enzymes. In addition, very often, there is a discrepancy between in vitro and in vivo results.

Table 8. In vitro protein digestibility of CF hydrolyzates.

Hydrolyzate	Digestibility (%)
Enzymatic hydrolysis	80.23 ^a \pm 0.20
Sequential hydrolysis (ultrasound and enzymatic steps)	100.00 ^b \pm 0.09

n = 3 repetitions per treatment; it means followed by different superscript lowercase letters indicate a significant difference between treatments at a 5% level by Tukey's test.

Thus, the strategy—the sequential hydrolysis composed of ultrasound and enzymatic steps—is a promising hydrolysis approach to produce CF hydrolyzates with high digestibility. Regarding antioxidant activity, for the sample concentration required for 50% radical inhibition (IC₅₀), when compared to the enzymatic hydrolysis (DPPH^{•+} IC₅₀ = 3.7 mg·mL⁻¹; ABTS^{•+} IC₅₀ = 1.5 mg·mL⁻¹), the sequential hydrolysis composed of ultrasound and enzymatic steps showed higher antioxidant activity (DPPH^{•+} IC₅₀ = 2.3 mg·mL⁻¹; ABTS^{•+} IC₅₀ = 0.25 mg·mL⁻¹), which, very likely, is related to antioxidant properties of peptides formed [38].

4. Conclusions

An environmentally friendly promising methodology for the production of chicken feather peptides was achieved. Thus, this approach is an interesting alternative to the

valorization of an abundant by-product. When compared to enzymatic hydrolysis, the strategy using a sequential hydrolysis with ultrasound and enzymatic steps reached a higher degree of hydrolysis, higher digestibility, and higher antioxidant activity. The sequential hydrolysis also produced unique dipeptides. Further investigation should be carried out on the identification of amino acids sequences, purification strategies, mainly membrane-based technology, and the biological application of the chicken feather peptides. It is worth noting that a similar strategy could be applied to other by-products (residue) composed of high protein content. The chicken feather peptides could be applied, mainly, into food, cosmetic, and pharmaceutical formulations.

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