

***In vitro* antioxidant properties and digestibility of chicken feather protein hydrolysates**

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Abstract

The *in vitro* antioxidant property and digestibility of chicken feather protein hydrolysate (CFPH) were evaluated in this study. The antioxidant property of CFPH obtained following chemical treatment of chicken feather waste involving precipitation with various acids (H₂SO₄, HNO₃, TCA and HCl) was determined via its scavenging action against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, iron reduction power and metal ion chelating activity. Data obtained showed that CFPH_{HNO₃} had the highest DPPH scavenging activity while CFPH_{TCA} exhibited the highest ferric (Fe³⁺) reduction potential. On the other hand, CFPH_{TCA}, CFPH_{H₂SO₄} and CFPH_{HNO₃} showed a similar capacity for Fe²⁺-chelation compared to CFPH_{H₂SO₄} with the least chelating potential. The *in vitro* protein digestibility of the CFPH of the various acids ranged from 62.30±1.0% (CFPH_{HNO₃}) to 73.10±1.3% (CFPH_{TCA}) and were significantly ($p < 0.05$) higher compared to the raw feather (23.80±0.5%). These results indicate that CFPH may be useful as antioxidants in animal feed formulations and also serve as additional source of essential nutrients in feeds.

1. Introduction

Feather wastes are often a source of environmental pollution. Thus, researches aimed at transforming these wastes into value-added products are warranted. The global annual contribution of solid waste in the form of feather is substantial. This is attributable to the rise in the global consumption of chickens (Jayathilakan *et al.*, 2012; Boland *et al.*, 2013). Feathers are very high in protein (84%) but have a very low digestibility (Akpor *et al.*, 2018). The principal protein in the feather is beta keratin, which is recalcitrant to enzymatic breakdown by animal, plant and numerous microorganisms (Onifade *et al.*, 1998; Zaghoul *et al.*, 2011), hence contributing to the low biodegradability of feathers. This low decomposition processes most often result in environmental pollution. Therefore, with the recent realities on the effects of climate change, and the call for more rigid regulations on refuse and waste disposal, new methods for handling feather wastes are required.

Recently, there has been an increased interest in the

search for natural antioxidants with less potential health hazard as an alternative to synthetic antioxidants. Consequently, research on the antioxidant property of agro-wastes has gained increased interest. Antioxidants in foods, in addition to their importance in animal health, are vital in the prevention of food deterioration (Fawolo *et al.*, 2014). Auto-oxidation process has been implicated in food deterioration (Carocho and Ferreira, 2013). The consumption of oxidized foods confers serious health challenges to the consumer and has been implicated in the pathogenesis of diseases such as ageing, cancer, diabetes, hypertension (Kanner, 2007). Bioactive peptides with high antioxidant activity have been extracted from enzymatically hydrolyzed feather keratin. Keratinous hydrolysates have been reported to demonstrate antioxidant activity especially in comparison to collagenous hydrolysates (Lasekan *et al.*, 2013). A report by Fakhfakh *et al.* (2011) showed that chicken feather hydrolysate obtained following the fermentation of feathers with the bacterium *Bacillus pumilus* A1 exhibited DPPH radical scavenging activity

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of 0.3 mg/mL after 48 hrs. In this context, the conversion of feather biomass into feather protein hydrolysates with potent antioxidant property would be an interesting possibility.

The choice of method for the hydrolysis of proteins most often is dependent on the source of the protein in question. Keratin from hair, horns, feathers, beaks or wool is most often hydrolyzed by treatment with acid, alkalis or microbial keratinases (Hou *et al.*, 2017). Therefore, the use of acids or alkalis in the hydrolysis of feather biomass is a very typical method used in the biomass transformation process (Tesfaye *et al.*, 2017; Akpor *et al.*, 2019). Such treatments have been found to also improve the solubility and susceptibility of feather protein to the action of proteolytic enzymes (Steiner *et al.*, 1983). Thus, chemical hydrolysis of chicken feather wastes using alkalis remains a viable option in the enhancement of the digestibility of feather either as feedstuff and food supplements. Information on the bioactivity of chemically hydrolyzed feather protein hydrolysate is scanty. Therefore, this study was designed to evaluate the *in vitro* antioxidant property and digestibility of alkaline-hydrolyzed chicken feather hydrolysate.

2. Materials and methods

2.1 Chicken feather waste

White-colored chicken feather waste was collected from the slaughterhouse of the Landmark University Commercial Farm (Omu-Aran, Nigeria).

2.2 Preparation of chicken feather protein hydrolysate

Chicken feathers were washed with detergent and 5% hypochlorite solution, rinsed thoroughly with a copious amount of water, and sun-dried. The dried feathers were ground into powder using a mechanical grinder. A total of 300 g of the powdered feathers was

weighed and soaked in acetone for 6 hrs and then dried before being extracted with a 1 M NaOH solution (wt/vol, 3:10) for 6 hrs at room temperature with constant stirring. Thereafter, the resulting mixture was filtered using a clean dry muslin cloth to remove unhydrolyzed feathers. The hydrolyzed feather solution was divided into four portions. The pH of each of the hydrolyzed feather solution was adjusted to neutral separately with 10% trichloroacetic acid (CFPH_{TCA}), 1 M H₂SO₄ (as CFPH_{H₂SO₄}), 1 M HNO₃ (as CFPH_{HNO₃}) and 1 M HCl (as CFPH_{HCl}) respectively. The resulting mixture was centrifuged (3000 × g) at 4°C for 10 mins discarding the supernatant thereafter. The obtained CFPH was dialyzed with cellulose tubes immersed in distilled water for 72 hrs while changing the water 3 times within 24 hrs. The dialyzed feather hydrolysate was freeze-dried to obtain chicken feather protein hydrolysate powder which was stored in a dried airtight container and at 4°C until it was required for further analysis. The procedures for the preparation of CFPH is shown in Figure 1.

2.3 Compositional analysis

The unprocessed chicken feathers and the respective acid CFPHs were analyzed for crude protein by the Kjeldahl method (Zhu *et al.*, 2010). Similarly, amino acid profile for both raw chicken feather and the respective acid CFPH was determined following hydrolysis with 6 M HCl (containing phenol) for 24 hrs at 115°C in glass tubes sealed under vacuum according to the method of Ravindran *et al.* (2005). Each analysis was carried out in triplicates.

2.4 Antioxidant assays

2.4.1 DPPH scavenging activity

The scavenging activity of the respective acid CFPH against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was estimated following the method of Bersuder *et al.* (1998) using butylated hydroxyanisole (BHA) as standard. Briefly, for the respective acid 1 mL of CFPH

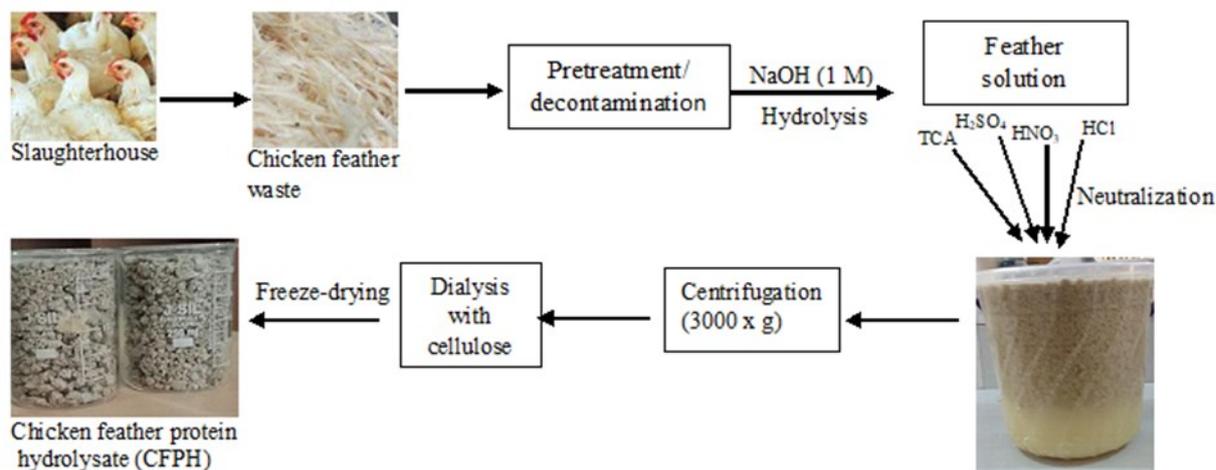


Figure 1. Flow chart for the extraction of chicken feather protein hydrolysate from waste feathers (CFPH)

corresponding to different protein concentrations (0.2 – 1.0 mg/mL) was added to 0.1 mL DPPH in ethanol. The resulting mixture was vortexed for 1 hr and kept at 25°C in the dark. Thereafter, the absorbance of the reaction mixture was taken at 517 nm. A blank in which distilled water was added in lieu of sample was run in the same way. A sample control in which ethanol was added in lieu of DPPH was also carried out for the respective CFPH. Each determination was carried out in triplicate. The DPPH radical scavenging activity was calculated in percentage according to the formula:

$$\text{Scavenging activity (\%)} = 1 - \frac{A_{\text{sample}} - A_{\text{sample control}}}{A_{\text{blank}}} \times 100$$

2.4.2 Fe^{3+} reducing activity

The Fe^{3+} reducing potential of the respective CFPH was estimated according to the method Yindirir *et al.* (2001). To a 2 mL of the respective CFPH at different protein concentrations (0.1 – 1.0 mg/mL) 2 mL phosphate buffer (0.2 mM, pH 6.6) and 2 mL potassium ferricyanide (1%) were added. The resulting mixture was incubated at 50°C for 20 mins before adding 2 mL of trichloroacetic acid (TCA, 10%) and then centrifuged at 1500 x g for 10 mins. To a 2 mL of the supernatant 2 mL of distilled water and 0.4 mL of ferric chloride (1%) were added. After 10 mins, the absorbance of the solution was taken at 700 nm. For the control, an equivalent volume of distilled water was added instead of the sample. Analysis for each sample was carried out in triplicates.

2.4.3 Metal (Fe^{2+}) chelating activity

The respective acid CFPH were evaluated for iron-chelating activity according to the methods described by Ebrahimzadeh *et al.* (2008). To 1 mL of the respective CFPH at different protein concentrations (0.2 – 1.0 mg/mL), 3.7 mL distilled water was added. Thereafter, 100 μL of 2 mM FeCl_2 was added. After 3 mins, the reaction was stopped by adding 200 μL of 5 mM ferrozine solution. The resulting mixture was shaken vigorously and left at 37°C for 10 mins before reading the absorbance at 562 nm. In the same way, a blank was run using distilled water in lieu of the sample. Analysis for each sample was done in triplicates. The iron-chelating activity was calculated in percentage according to the formula:

$$\text{Iron chelating activity (\%)} = 1 - \frac{A_{\text{sample}} - A_{\text{sample control}}}{A_{\text{blank}}} \times 100$$

2.4.4 Determination of *in vitro* protein digestibility

The *in vitro* protein digestibility of the respective CFPH was evaluated using the multi-enzyme solution according to the method described by Manjula and John (1991) with little modifications. A known weight of the respective CFPH containing 16 mg nitrogen was digested with 1 mg pepsin dissolved in 15 mL of HCl

(0.1 M) at room temperature for 2 hrs. The reaction was inhibited by adding 15 mL TCA (10%). The resulting mixture was filtered using Whatman No. 1 filter paper. Thereafter the nitrogen content of the TCA-soluble fraction was determined using the micro-Kjeldahl method and the *in vitro* protein digestibility was estimated using the equation:

$$\text{In vitro protein digestibility (\%)} = \frac{\text{Content of protein released upon the digestion of 1 g of sample}}{\text{Content of 1 protein of 1 g of sample before digestion}}$$

2.5 Statistical analyses

The results are presented as the means \pm SD of triplicate biological assays. The statistical analysis was by One-way analysis of variance (ANOVA) followed by Turkey's Multiple Comparison using SPSS version 20. $P < 0.05$ was considered significant. All graphs were plotted using Graph Pad Prism.

3. Results

3.1 Proximate composition

The crude protein content of CFPH had significantly higher crude protein (88.6 \pm 0.04%) compared with the raw feather (71.8 \pm 0.1%). There was a significant decrease in methionine, lysine, cysteine and histidine level in the CFPH compared to the raw chicken feather (Table 1).

3.2 Antioxidant activity

3.2.1 DPPH scavenging activity

The DPPH scavenging activity of the respective acid CFPH was observed to be concentration dependent. CFPH_{HNO₃} exhibited the highest scavenging activity, followed by CFPH_{H₂SO₄} while CFPH_{HCl} showed the least activity (Figure 2).

3.2.2 Reducing power assay

CFPH_{TCA} showed significantly higher ferric reduction potential across all concentrations compared to CFPH of the other acids. No significant difference in ferric reduction activity was observed between CFPH_{H₂SO₄}, CFPH_{HNO₃} and CFPH_{HCl} (Figure 3).

3.2.3 Metal chelating activity

The metal chelation activity of the respective acid CFPH was observed to be concentration dependent. No significant difference in iron-chelating activity was observed between CFPH_{TCA}, CFPH_{H₂SO₄} and CFPH_{HNO₃} but the CFPH of the 3 acids exhibited significantly iron-chelating activity compared to CFPH_{HCl} (Figure 4).

3.2.4 *In vitro* digestibility

The *in vitro* protein digestibility recorded for the hydrolysates showed that CFPH_{HCl} > CFPH_{HNO₃} >

Table 1. Chemical composition of raw chicken feather and the respective acid chicken feather protein hydrolysate (CFPH)

	Ratio of fish meal to chicken feather protein hydrolysate					
	100:0	80:20	60:40	40:60	20:80	0:100
Proximate composition (%)						
Moisture	7.3±1.0 ^a	9.0±2.0	9.0±1.5	9.0±1.3	8.0±0.3	9.0±0.7
Crude protein	24.42±1.1 ^a	23.83±0.2 ^a	21.12±0.5	20.96±1.2	22.16±0.4	23.86±0.0
Nitrogen-free extract ¹	55.88± 5.2 ^a	57.17±3.8 ^a	61.08±5.5 ^a	61.14±2.1 ^a	60.34±5.8 ^a	58.04±4.9 ^a
Energy (kcal/100 g)	358.4 ^a	346.5 ^a	349.5 ^a	349.1 ^a	349.8 ^a	343.8 ^a
Amino Acid Composition						
Lysine	2.56 ^a	0.58 ^b	0.42 ^{b,c}	0.33 ^c	0.33 ^c	0.31 ^c
Threonine	4.50	4.52	4.38	4.35	4.35	4.33
Cysteine	1.02 ^a	2.85 ^a	3.27 ^b	3.33 ^b	3.50 ^b	3.30 ^b
Leucine	6.88	6.55	7.61	7.53	7.83	7.82
Isoleucine	4.58	4.55	4.38	4.53	4.52	4.50
Tryptophan	2.56 ^a	1.01 ^b	0.62 ^c	0.55 ^c	0.48 ^c	0.48 ^c
Methionine	2.79 ^a	1.53 ^b	0.88 ^c	0.85 ^c	0.81 ^c	0.77 ^c
Phenylalanine	5.53	5.50	5.33	5.35	5.30	5.22
Histidine	3.03 ^a	2.82 ^a	0.73 ^b	0.65 ^b	0.62 ^b	0.63 ^b
Valine	9.55	9.33	9.05	9.03	8.93	8.88
Arginine	4.83	4.85	4.55	4.33	4.55	4.52
Serine	12.58	12.80	12.85	13.20	13.20	13.05
Glycine	10.20	10.21	9.55	9.80	9.85	9.85

Values are given as mean ± SD of triplicate determinations. Values in the same row carrying different superscripts are significant (P<0.05).

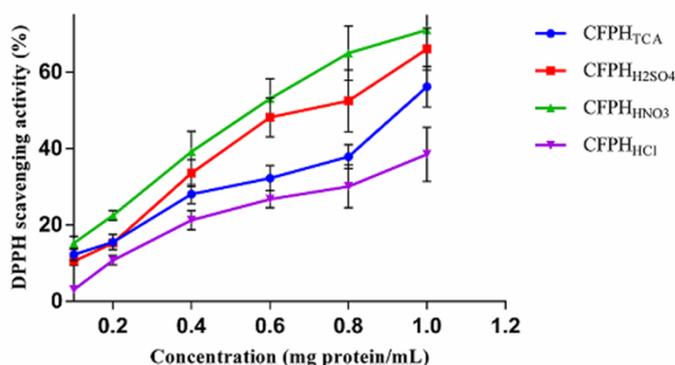


Figure 2. DPPH scavenging activity of the respective acid CFPH at different protein concentrations. Values are means ± SD of three determinations. Note: CFPH, chicken feather protein hydrolysate

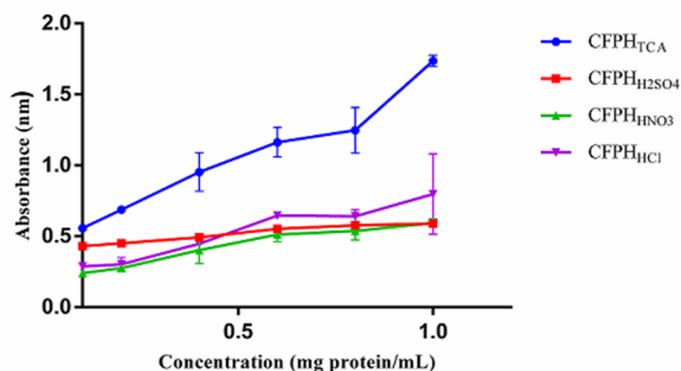


Figure 3. Ferric reducing activity of the respective CFPH at different protein concentrations. Values are means ± SD of three determinations. Note: CFPH, chicken feather protein hydrolysate

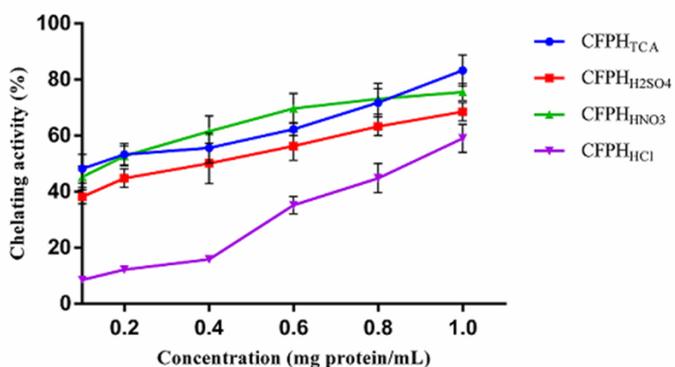


Figure 4. Iron chelating activity of the respective acid CFPH at different protein concentrations. Values are means ± SD of three determinations. Note: CFPH, chicken feather protein hydrolysate

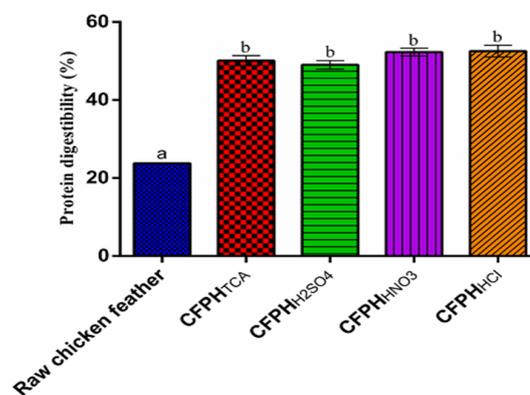


Figure 5. In vitro protein digestibility of the different chicken feather protein hydrolysates. Values are means ± SD of three determinations. Note: CFPH, chicken feather protein hydrolysate

CFPH_{TCA} > CFPH_{H₂SO₄} with values 52.5%, 52.3%, 50.1% and 49.0% respectively. The differences in digestibility across the different hydrolysates were not significant ($p>0.05$) but were significantly higher than that of the raw feather (Figure 5).

4. Discussion

In the present study, CFPH was demonstrated to show antioxidant activity *in vitro* through its scavenging action against DPPH, Fe³⁺ reduction potential and iron-chelating activity. These results agree with the report of a study Je *et al.* (2007) in which protein hydrolysate obtained from bullfrog muscle was reported to demonstrate antioxidant activity using DPPH scavenging and ferrozine assays. Similarly, Chan *et al.* (1994) showed that meat dipeptide carnosine antioxidant action was as a result of its chelation activity against prooxidant metals. In addition, hydrolysate obtained from porcine myofibrillar via enzymatic hydrolysis was reported to possess excellent DPPH scavenging and metal chelation activities (Saiga *et al.*, 2003). The antioxidant activity of protein hydrolysate has been attributed to the action of peptides (Gomez-Guillen *et al.*, 2011). Chemical or enzymatic hydrolysis disrupts protein tertiary structure thus enhancing the solvation properties of its amino acid residues and consequently its antioxidant activity. The resulting peptides protein hydrolysis have been demonstrated to show enhanced antioxidant activity compared to intact proteins. The excellent antioxidant potential of proteinaceous supplements has enabled their inclusion in foods to retard or inhibit the oxidation of foods. The antioxidant action of free of protein hydrolysates involves such mechanisms as deactivation of reactive oxygen species, reduction of hydroperoxides, chelation of prooxidant metallic ions, and changes in the physical properties of food systems (Elias *et al.*, 2008; Tang *et al.*, 2009).

The high amounts of sulfur-containing amino acids, cysteine have been indicated to account for the antioxidant activity of feather keratin. For instance, in a study by Ohba *et al.* (2003), enzymatic hydrolysate obtained from a mixture of horn, hoof and chicken feather was demonstrated to show enhanced antioxidant activity. In another related study, Fakhfakh *et al.* (2013) reported also that protein hydrolysate obtained from chicken feather fermented with the bacterium *Bacillus pumilus* A1 showed strong antioxidant activity.

Data from this study revealed that the use of alkalis in the hydrolysis of chicken feathers to obtain CFPH significantly improved the digestibility of feather *in vitro*. This is in agreement with the report of Steiner *et al.* (1983) in which feathers treated with varying

concentrations of NaOH or H₃PO₄ showed improvement *in vitro* pepsin digestibility. In a related study by Papdopoulos (1985) broiler feathers with various concentrations of NaOH or maxatase showed increased solubility and susceptibility to digestion by proteolytic enzymes. It could thus be argued that treatment with NaOH or enzyme weakens and exposes the disulfide linkages in feather keratin backbone thus increasing the solvation property of its amino acid residues culminating in increased solubility of CFPH and enhanced susceptibility to proteolytic digestive enzymes vis-à-vis its digestibility and utilization as the growth substrate.

6. Conclusion

Results on data generated in this study, alkaline hydrolyzed chicken feather protein hydrolysate exhibited excellent antioxidant property through its DPPH scavenging activity, iron-reducing property and metal ion chelating potential. In addition, significant improvement in the *in vitro* digestibility of chicken feather protein hydrolysate was demonstrated due to alkaline hydrolysis of the chicken feather. Based on these results, the inclusion of chicken feather hydrolysate in animal feed formulations could be advisable not only to preserve the integrity of the feedstuff but also to enhance the functional attributes of the feed as well as an additional source of essential nutrients.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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