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Antioxidant activity of peptides extracted from chicken intestine hydrolysate

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Abstract
Chicken intestine was hydrolysed by protease P food grade fungal enzyme. The protein hydrolysate solution was filtered and separated into small molecular weight fractions by ultrafiltration at 4 °C using 10kDa molecular weight cut-off and 3kDa molecular weight cut-off to enrich specific hydrolysate fractions. This permeate was defined as small peptides with molecular weight less than 10,000 Da and 3000 Da. The filtrate thus obtained was assessed for antioxidant *in vitro* DPPH radical scavenging assay. Parameters like filterate, concentrate, and total recovery of hydrolysate was studied after ultrafilteration. The filtrate recovery of protein hydrolysate was 47.01 ± 0.2 per cent, concentrate recovery of protein hydrolysate was 5.12 ± 0.45 per cent, total recovery of protein hydrolysate was 52.14 ± 0.2 per cent. The inhibitory concentration IC_{50} value by DPPH radical scavenging assay of hydrolysate was 1.45 ± 00 mg/ml, bioactive peptides (< 10 kDa) was 0.15 ± 00 mg/ml and bioactive peptides (<3kDa) was 1.90 ± 0.8 mg/ml. Then it was compared with the standard butylated hydroxyl toluene whose IC_{50} value 0.23 ± 0.3 mg/ml. The bioactive peptides was having lower IC_{50} value than hydrolysate. So, lower IC_{50} value indicate higher potency of the extracted peptides when compared to the standard butylated hydroxyl toluene. Antioxidant bioactive peptides extracted from chicken intestine had the molecular weight between 9.4 kDa to 9.8 kDa. Thus the byproduct from chicken industry i.e chicken intestine can be utilized by transforming in to antioxidant bioactive peptides as alternatives to synthetic antioxidants used in pet food.

Keywords: chicken intestine, enzymatic hydrolysis, protease p, bioactive peptides, antioxidant peptides

Introduction
Meat by-products (trimmings and mechanically recovered meat, collagen, blood, offals) are generally very rich in proteins. Proteins associated with meat industry by products constitute more than one eighth of total protein in the lean meat (Webster *et al.*, 1982) [12]. Bioactive peptides are about 2-30 amino acids short peptides in length. They are inactive within in sequence and exert bioactive functions on hydrolysis. Meat by-products derived peptides have antioxidant, antimicrobial, antithrombotic, ACE-I inhibitory activity, immuno modulatory activity, lipid lowering activity and anti diabetic activity. Peptides derived from meat proteins offer a promising approach to prevent, control and even treat lifestyle-related diseases through regulated diet. Bioactive peptide fits into the trends in the development of innovative functional products and nutraceuticals. Hence, an attempt is made to extract the antioxidant bioactive peptides from chicken intestine hydrolysate.

Materials and Methods
Samples of chicken intestine were collected from local retail outlets. The chicken intestines were collected from retail outlets were ice packed and immediately brought to the Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Chennai of Commercially available food grade fungal enzyme, protease P "Amano"6 having not less than 60,000 u/g proteolytic activity, was procured from M/s.Amano Pharmaceutical Co. Ltd., Japan.

Methods
The protein hydrolysate was prepared according to the method of Bhaskar *et al.*, (2007) [1]. The chicken intestine of about 500 gms for 6 trails was used after proper cleaning of intestine in running tap water to remove the intestinal contents, dipped in boiling water for 5 minutes and then were cut in to small pieces. These small pieces were sterilized at 121 °C under 15 lbs pressure for 15 minutes. The sterilized chicken intestinal pieces were then cooled and minced

in a Waring blender for 5 minutes, followed by centrifugation at 10,500 rpm for 30 minutes at 4 °C. After centrifugation, the contents were separated in to three phases in which the top layer contain fat, mostly of middle layer water and protein rich sediment at the bottom. Both the fat and water layers were discarded and only the protein rich sediment was collected and used for further processing.

Enzymatic hydrolysis of the protein rich sediment

The protein rich sediment was mixed with equal quantity of water (w/v), added with 1.0% fungal protease P (Phycomycetes enzyme) used for hydrolysis at 43±1 °C for 90 min in a hot water bath. After the period of time, hydrolysis was stopped by heating the mixture kept at 85 °C for 5 minutes. The hydrolysate was centrifuged at 11,000 rpm for 20 minutes at 15 °C and the supernatant was collected. The collected supernatant containing protein hydrolysate was used for further studies.

Ultrafiltration of the Protein Hydrolysate

The protein hydrolysate solution was filtered and separated into small molecular weight fractions by ultrafiltration at 4 °C using 10kDa molecular weight cut-off and 3kDa molecular weight cut-off to enrich specific hydrolysate fractions. This permeate was defined as small peptides with molecular weight less than 10,000 Da and 3000 Da. The filtrate thus obtained was assessed for bioactivity using In-vitro DPPH radical scavenging assay.

***In vitro* assays for determination of bio-activity**

Antioxidant activity of protein hydrolysate and extracted bioactive peptides

Antioxidant activity was measured using DPPH radical scavenging assay. The antioxidant activity of protein hydrolysate and extracted bioactive peptides were tested and compared with butylated hydroxyl toluene which was taken as standard.

2,2-Diphenyl-1-Picrylhydrazyl Radical Scavenging Activity

Scavenging activity on DPPH free radicals by the samples were assessed according to the method reported by Sunitha *et al*. (2016) [10] with slight modifications.

a. Preparation of DPPH solution

4.3mg of 2,2-diphenyl-1-picrylhydrazyl was dissolved in 3.3ml methanol and was protected from light by covering the test tubes with aluminium foil.

b. Preparation of standard solution

0.2 g of Butylated hydroxyl toluene was dissolved in 10ml of methanol to get 20mg/ml concentration of stock solution. Different concentration of BHT was made from stock solution (0.2, 0.4, 0.8, 1.2, 1.6 and 2mg/ml)

c. Protocol for estimation of DPPH scavenging activity and IC_{50} value

1. 150µl DPPH solution was added to 3ml methanol and absorbance was taken immediately at 516nm for control reading.
2. Different volume levels of test samples (10, 20, 40, 60, 80 and 100µl) were screened each dose level was made up to 200 µl by dilution with methanol in test tubes.
3. All levels of test samples with different concentration were diluted with 3ml of methanol.
4. 150µl of DPPH solution was added to each test tubes.
5. Absorbance was taken at 516 nm in in UV-Vis spectrometer after 15 minutes using methanol as a blank.

The percentage of radical scavenging activity (RSA) was assessed and IC_{50} value was calculated.

$$(\%)\ RSA = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100$$

The effective concentration of sample required to scavenge DPPH radical by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between per cent inhibition and concentrations.

Characterisation of bioactive peptides

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – Page)

SDS-PAGE was done in Mini Vertical Gel Electrophoresis unit (SDS-PAGE apparatus, Model Regular-Mini Vertical, ORANGE^{TM}, Chennai) available in the Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Chennai-7. Chemicals for SDS PAGE were (Acrylamide bisacrylamide solution 29:1), SDS, Tris buffer 6.8 and 8.1, Broad range protein molecular marker, TEMED, Ammonium per sulphate, Tris glycine SDS running buffer (10X) bought from Medox Biotech Pvt. Ltd., and stored in appropriate temperature for further use.

Preparation of Tris - Glycine SDS BUFFER Solution

One litre of running tank buffer was prepared by mixing 100 ml of stock solution with 900 ml of distilled water and stored in bottle at room temperature for further use.

Ammonium per Sulphate Solution and SDS Solution Preparation

One gram of SDS was mixed with 9 ml of distilled water in a 15 ml measuring centrifuge tube and then the volume was made upto 10 ml using distilled water. This solution was then stored at room temperature for further use. For preparing 10% ammonium per sulphate, 500 mg of ammonium per sulphate was taken and mixed with 4 ml of distilled water, and then the volume was made to 5 ml using distilled water and stored at 4 °C for further use.

Composition of Separating Gel (15%)

S. No	Chemicals	Quantity
1.	Acrylamide bisacrylamide mix (29:1)	5 ml
2.	Distilled Water	2.2 ml
3.	1.5 M Tris buffer 8.8	2.6 ml
4.	10% SDS (w/v)	0.1 ml
5.	10% Ammonium per sulphate (w/v)	100 µl
6	Temed	10 µl

Composition of Stacking Gel (5%)

S. No	Chemicals	Quantity
1.	Acrylamide bisacrylamide mix (29:1)	0.67 ml
2.	Distilled Water	2.975 ml
3.	0.5 M Tris buffer pH 6.8	1.25 ml
4.	10% SDS (w/v)	0.05 ml
5.	10% Ammonium per sulphate (w/v)	0.05 ml
6.	Temed	0.005 ml

Composition of Staining Solution

S. No	Chemicals	Quantity
1.	Coomassie brilliant blue R-250	1.25 g
2.	Methanol	225 ml
3.	Glacial acetic acid	50 ml
4.	Distilled water	225 ml

Composition of Destaining Solution

S. No	Chemicals	Quantity
1.	Methanol	150 ml
2.	Glacial acetic acid	50 ml
3.	Distilled water	300 ml

Preparation of Gel for SDS - Page

1. Separating Gel Preparation

The casting frames (clamp two glass plates in the casting frames) were set on the casting stands. The gel solution (as described above) was prepared in a separate 15 ml centrifuge tubes. The solution was swirled gently but thoroughly. Appropriate amount of separating gel solution (listed above) was pipetted into the gap between the glass plates. The top of the separating gel was made horizontal by filling in water (either isopropanol) into the gap until it overflows. The set up was left undisturbed for 20-30 minutes to gelate.

2. Stacking Gel Preparation

The water was discarded from separating gel after gelation and then stacking gel was layered over separating gel until it overflowed. The well-forming comb was inserted gently without trapping air under the teeth and left for 20-30 min to gelate. After complete gelation of the stacking gel, the comb was removed. The glass plates were taken out of the casting frame and set in the buffer tank. The running buffer (electrophoresis buffer) was poured into tank until the buffer surface reached the required level. The prepared samples were loaded in wells and taken care not to overflow. The protein marker was loaded into the first lane. The electrophoresis tank was connected to the electrodes and an appropriate voltage was set and made to run.

3. Preparation of Sample for SDS-Page

The protein fractions were prepared for gel electrophoresis by mixing 20µl of each sample with 20µl glycine sample buffer. The mixer was boiled for 10 minutes in boiling water and the resultant mixer was added with an equal volume of sample buffer (0.5M Tris-HCl, pH 6.8 containing 4% (w/v) SDS and 20% (v/v) glycerol).

Electrophoresis and Imaging

SDS–PAGE was done according to the method of Laemmli (1970) [6] with slight modification using 15% stacking gel and a 5% resolving gel prepared in the lab with a constant current of 50 milliampere (mA). 20 µl of the sample was loaded in each well. The high molecular weight markers were used to estimate the molecular weight of the bands. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 6.8% (v/v) glacial acetic acid for overnight (12 hours) and destained using 7.5% (v/v) of glacial acetic acid and 5% (v/v) methanol for about 9 hours with a change of solution every 3 hours. The semi quantitative analysis of band intensity was done using the Gel Doc EZ Imager with the Image Lab 3.0 software (Bio-Rad Laboratories, Inc.).

Statistical Analysis

The data was subjected to statistical analysis in SPSS (version 2.0) software with mean ± SE.

Results and Discussion

Ultrafilteration of the Hydrolysate

1. Filtrate Recovery

The mean ± SE values of filtrate recovery of protein hydrolysate was
47.01 ± 0.2 per cent and ranged from 46.57 to 47.93 per cent were presented in the table 1.

2. Concentrate Recovery

The mean ± SE values of concentrate recovery of protein hydrolysate was 5.12 ± 0.45 per cent and ranged from 3.37 to 6.7 per cent were presented in the table 1.

3. Total Recovery

The mean ± SE values of total recovery of protein hydrolysate was 52.14 ± 0.2 per cent and ranged from 51.3 to 53.05 per cent were presented in the table 1. Jamdar *et al.* (2005) [3] had a total recovery of 66.6 per cent from autolysate of chicken intestine. while Picot *et al.* (2010) [8] obtained recovery rate of 69% from north atlantic fish hydrolysate permeate for 4 kDa molecular weight cut off.

Table 1: Mean ± SE values of recovery rate of low molecular weight peptides by Ultrafiltration

Parameters	Recovery %
% Filtrate recovery	47.01 ± 0.2
% Concentrate recovery	5.12 ± 0.45
% Total recovery	52.14 ± 0.2

***In vitro* Assays for Determination of Bioactivity**

DPPH Radical Scavenging Assay for Antioxidant Activity

The mean ± SE values of IC_{50} value of hydrolysate was 1.45 ± 00 mg/ml. The mean ± SE values of bioactive peptides (< 10 kDa) was 0.15 ± 00 mg/ml and bioactive peptides (<3kDa) was 1.90 ± 0.8 mg/ml. Then it was compared with the standard butylated hydroxyl toluene whose IC_{50} value 0.23 ± 0.3 mg/ml were presented in the table 8 and figures 2, 3,4,5 and 6. The test of significance revealed that the DPPH antioxidant activity between hydrolysate and bioactive peptides was highly significant (P<0.01). However, the bioactive peptides was having lower IC_{50} value than hydrolysate. So, lower IC_{50} value indicate higher potency of the extracted peptides when compared to the standard butylated hydroxyl toluene. Escudero *et al.* (2013) [2] had dissimilar result of IC_{50} value of 1.5 mg/ml in the Spanish dry cured exhibited by DPPH radical scavenging assay.

Jamdar *et al.* (2012) [4] observed similar results in antioxidant activity of Poultry Visceral Protein Hydrolysate (PVPH). PVPH exhibited excellent DPPH radical scavenging activity at peptide concentration of 0.2 and 2.0 mg/mL, respectively. Lee *et al.* (2012) [7] had similar results in peptide fractions of < 5KDa fractions of duck hydrolysate was having highest hydroxyl radical scavenging activity exhibiting an IC_{50} value of 0.53 mg/ml by using DPPH antioxidant assay. They also reported that the antioxidant properties of gelatin from duck skin and found that the extracted peptides was having an IC_{50} value of 0.63 mg/ml. on contrary to our results Sun *et al.* (2012) [9] observed the antioxidant properties of chicken breast protein hydrolysate that the extracted peptides had an IC_{50} value of 1.28 mg/ml by using DPPH antioxidant assay.

The mean ± SE values of IC_{50} value of hydrolysate, bioactive peptides (< 10 kDa), bioactive peptides (<3kDa) and standard butylated hydroxyl toluene were presented in table 2.

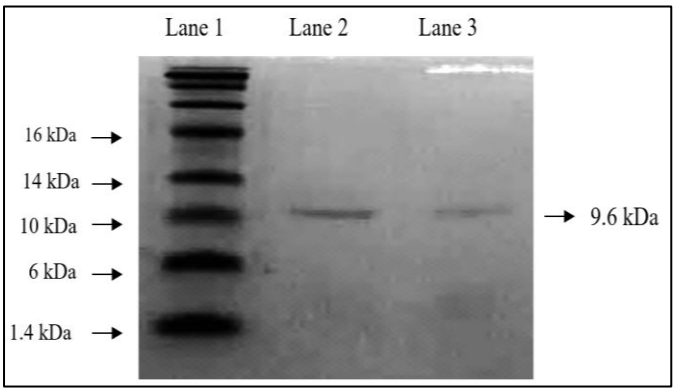
Table 2: Mean ± SE values of DPPH antioxidant assay in hydrolysate and low molecular weight peptides with standard butylated hydroxyl toluene

Antioxidant activity	IC_{50} value
Butylated hydroxyl toluene	0.23 ± 0.3
Protein hydrolysate	1.45 ± 00**
Bioactive peptides (<10 KDa)	0.15 ± 00**
Bioactive peptides (<3 KDa)	1.90 ± 0.8**

NS -Not Significant * - Significant (P<0.05) difference** - Highly significant (P<0.01) differenceMeans bearing different superscripts in the same row differ significantly

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS - Page)

The SDS-PAGE patterns of bioactive peptides are shown in the Plate 1. The SDS-PAGE showed bands in lane 1 representing the polypeptide protein marker which recorded five bands with the molecular weight of 16, 14, 10, 6, 1.4 kDa in sequential order; whereas the extracted bioactive peptides were in lane 2, lane 3 which depicted a band with molecular weight of between 9.4 kDa to 9.8 kDa. In the present study, bioactive peptides extracted from chicken intestine had the molecular weight between 9.4 kDa to 9.8 kDa. Similar results were obtained Kim *et al* (2009) [5] who characterized the antioxidant peptides from venison with molecular weights of 9.8 kda and 11 kda and also Thiansilakul *et al.* (2007) [11] extracted anti-oxidant peptides from round scad muscle hydrolysate with a molecular mass of 9 kDa.



Lane 1: Polypeptide protein marker
Lane 2: Extracted bioactive peptides
Lane 3: Extracted bioactive peptides

Plate 1: Molecular pattern of bioactive peptides on SDS- Poly Acrylamide Gel Electrophoresis (SDS-PAGE) – 15% separating gel

Conclusion

Bioactive peptides extracted from chicken intestinal hydrolysate was having antioxidant activity in *in vitro* DPPH radical scavenging assay. Chicken intestines from poultry industry can be efficiently utilized for extraction of bioactive peptides. It can be used as alternatives for synthetic antioxidants. Further studies should be done in live animals regarding antioxidant activity and there may be various factors to be assessed about their mechanism of action before including in pet food and other applications.

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