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Assessing the antioxidant activity of peptides obtained from chicken intestine hydrolysate in canine pellet food using DPPH radical scavenging assay

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Abstract
Chicken intestine was hydrolysed by protease P food grade fungal enzyme. Chicken intestinal hydrolysate was ultrafiltered using 10 kda molecular weight cut-off and the filtrate is freeze dried. Then the low molecular weight peptides is incorporated in canine pellet feed at 5%, 6% and 7% levels. Canine pellet feed with different levels of incorporated peptides was assessed for antioxidant activity using DPPH radical scavenging assay. The mean±SE values of IC_{50} value for anti-oxidant activity of bioactive peptide incorporated at different levels of 5%, 6% and 7% in canine pet food were 1.80±0.4 mg/ml, 1.41±0.6 mg/ml and 0.20±0.70 mg/ml respectively.
These values were compared with the IC_{50} value for anti-oxidant activity of canine pet food prior to addition of bioactive peptides which was 1.84±0.4 mg/ml which is taken as standard. The test of significance revealed that there was a highly significant ($p<0.01$) difference between the IC_{50} values of canine pet food incorporated with different levels (5%, 6% and 7%) of bioactive peptides. The canine pet food with 7% incorporated bioactive peptide had lower IC_{50} value than the other levels indicating that the potency of the extracted peptides has been exhibited in the incorporated canine pet food.

Keywords: Chicken intestine, bioactive peptides, antioxidant bioactive peptides, canine pellet food, DPPH radical scavenging assay

Introduction
Bioactive protein hydrolysates could also be a potential source of natural and safer antioxidants Ruijia Hu *et al.* (2020) [6]. Lipid oxidation is a major cause of quality deterioration during processing, handling, and storage of high-fat/oil foods or ingredients. C.J. Mussinan *et al.* (1998) [4]. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), and ethoxyquin (EQ) are commonly used in various food and feed products. However, it has been reported that such antioxidants possibly increase health risks due to their toxicity and carcinogenicity C.F. Oliveira *et al.*,(2014) [3], F. Shahidi *et al.*,(2008) [5]. In this research work chicken intestine hydrolysate was used to study the antioxidant bioactive properties in canine petfood using DPPH radical scavenging assay.

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. The protein hydrolysate was prepared according to the method of Bhaskar *et al.*, (2007) [2]. 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 10 kDa molecular weight cut-off to enrich specific hydrolysate fractions. This permeate was defined as small peptides with molecular weight less than 10,000 Da. The filtrate thus obtained was freeze dried and incorporated in canine pet food for assesing bioactivity using In-vitro DPPH radical scavenging assay.

Incorporation of extracted bioactive peptides in canine pet food

Based on the nutrient specifications and recommendations given by the AAFCO (2014) for the adult dog a maintenance diet, supplemented with choline chloride was formulated and canine pet food was prepared by the Department of Animal Nutrition, Madras veterinary college, Chennai – 07.

The ingredients *viz.*, maize, wheat bran, soybean meal, sunflower oil cake, poultry by-product meal, vegetable oil and choline chloride were purchased from the manufacturers in required quantities as given in composition Table 1. The ingredients were dried and ground properly, and stored in a hygienic manner for the preparation of pet food. All the ingredients including oil were mixed thoroughly and 10% of water was added gradually in the mixer before extrusion.

Table 1: Composition of canine pet food

Ingredients	Inclusion level %
Wheat bran	58.00
Soybean meal	2.00
Sunflower oil cake	5.50
Poultry by-product meal	22.00
Vegetable oil	11.00
Iodized salt	0.60
Trace mineral mixture	0.50
Choline chloride	0.226
Vit AD3EK	0.053
Vit B-Complex	0.030
Vit E & Se	0.008
Liver tonic	0.050
Toxin binder	0.033
Total	100.00

One gram of vitamin AB2D3K supplement contained 82,500 I.U. of vitamin A, 50 mg of vitamin B2, 12,000 I.U. of vitamin D3 and 10 mg of vitamin K. one gram of B-complex supplement contained 8 mg of vitamin B1, 16 mg of vitamin B6, 80 mcg of vitamin B12, 8 mg of vitamin E, 8 mg of folic acid, 8 mg of calcium pantothenate, 120 mg of niacin, 100 mcg of selenium. Each 200 g of vitamin E and Se supplement contained 20 mg of vitamin E and 200 mg of selenium.

The materials were extruded through BTPL twin screw extruder (Model – TSE 002, Kolkata, India) with the extruder temperature fixed at 124 °C and the prepared food was conveyed through pneumatic conveyer to the drier, where the product was dried at 80 °C for two hours. Canine pet food was prepared by the above-said procedure and was cooled and packed in LDPE bags and stored at room temperature (30.16±1.26 °C). Canine pet food pellets were ground by using grinder in to fine powder. Bioactive peptides obtained from the chicken intestine samples were incorporated at different inclusion level of 5%, 6%, and 7% bioactive peptides in freeze dried powder form to make 100 gms of canine pet food and mixed thoroughly using mixer. The bio-activity assay of the bioactive peptides incorporated canine pet food samples at different levels of incorporation was carried out.

DPPH radical scavenging activity assay of bioactive peptides incorporated canine pet food at different incorporation levels

DPPH radical Scavenging activity were assessed for different inclusion level of bioactive

Peptides in canine pet food according to the method reported by Sunitha *et al.* (2016) [7] with slight modifications.

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) [7] 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. 150 µl 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 100 µl) 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. 150 µl 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.

The dried bioactive peptides powder extracts was dissolved in methanol. 5 mg/ml of pet food with bioactive peptides powder was dissolved in 0.5ml of methanol and was taken as stock solution for preparing different concentrations of test sample in the form of liquid. DPPH radical scavenging assay (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotted between % inhibition and concentrations.

Statistical analysis

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

Results and Discussion

DPPH assay among different incorporation levels in canine pet food. The mean±SE values of IC_{50} value for anti-oxidant activity of bioactive peptide incorporated at different levels of 5%, 6% and 7% in canine pet food were 1.80±0.4 mg/ml, 1.41±0.6 mg/ml and 0.20±0.70 mg/ml respectively as given in table 2.

These values were then incorporated with the IC_{50} value for anti-oxidant activity of canine pet food prior to addition of bioactive peptides which was 1.84±0.4 mg/ml. The test of significance revealed that there was a highly significant (p<s0.01) difference between the IC_{50} values of canine pet food incorporated with different levels (5%, 6% and 7%) of bioactive peptides. The canine pet food with 7% incorporated bioactive peptide had lower IC_{50} value than the other levels indicating that the potency of the extracted peptides has been exhibited in the incorporated canine pet food.

Table 2: Mean±SE values of DPPH antioxidant assay among different incorporation levels of extracted peptides in pet food

Antioxidant activity	IC $_{50}$ value
Pet food	1.84±0.4
5% incorporated pet food	1.80 ^{c}±0.5
6% incorporated pet food	1.41^{b}±0.6
7% incorporated pet food	0.20 ^{a}±0.70

NS -Not Significant

* - Significant (p<0.05) difference

** - Highly significant (p<0.01) difference

Means bearing different superscripts in the same row differ significantly

The results of DPPH radical scavenging activity among different incorporation levels were compared with standard pet food which was taken as control. Among different levels of incorporation 7 per cent inclusion was found to have more antioxidant activity with an IC $_{50}$ value of 0.20±0.70 when compared with other inclusion levels of 5 per, 6 per cent and the control having an IC $_{50}$ value of 1.80±0.5 mg/ml, 1.41±0.6 mg/ml and 1.84±0.4 mg/ml.

Thus, the study revealed that incorporation of bioactive peptides at 7% level had better antioxidant activity than the other levels. In contrast, Zimmerman *et al.* (1996) [8] suggested an incorporation level of 6% bioactive peptides in post weaning diet of piglets.

Conclusion

Thus chicken intestine can be utilized for inclusion in pet food. It reveals that bioactive peptides obtained from chicken intestinal hydrolysate was having antioxidant activites in DPPH radical scavenging assay. Further studies should be done in canine feeding trail regarding its application and action in live animal. Thus byproduct of chicken processing plant can be utilized as functional food for canine instead of rendering.

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