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Assessing the antidiabetic activity of peptides obtained from chicken intestine hydrolysate in canine pellet food using alpha amylase inhibitory assay

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Abstract
Chicken intestine was hydrolysed by protease P food grade fungal enzyme. Chicken intestinal hydrolysate was ultrafiltered using 10kda 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 antidiabetic activity using α-amylase inhibitory assay. The mean±SE values of IC_{50} value for anti-diabetic activity of bioactive peptide incorporated at different levels of 5%, 6% and 7% in canine pet food were 59.26±4.4 mg/ml, 364.28±13 mg/ml and 163.57±5.04 mg/ml respectively.
These values were then compared with the IC_{50} value for anti-diabetic activity of canine pet food prior to addition of bioactive peptides. Which was taken as control is 1313.85±52.8. The test of significance revealed that there was a highly significant difference between the different levels of bioactive peptides incorporated canine pet food. The 5% inclusion level was found to have less IC_{50} value of 59.26±4.4 µg/ml and better alpha amylase inhibition activity. The canine pet food with 5% incorporated bioactive peptides 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, anti-diabetic bioactive peptides, canine pellet food, alpha amylase inhibitory assay

Introduction
Bioactive protein hydrolysates could be a potential source of functional foods. Rand *et al.* (2004) [5] and Case *et al.* (2011) [3] suggested that Diabetes mellitus (DM) is a serious metabolic disease that is rising in prevalence worldwide in both humans and pets. It is characterized by persistent hyperglycemia and is caused either by inadequate insulin secretion from pancreatic beta cells, impaired insulin signaling, or both. DM most frequently diagnosed endocrine disorders in dogs and cats. Oligosaccharides were hydrolysed into monosaccharides by alpha – glucosidase and were transferred in to the blood stream. The inhibition of the enzymes α – amylase and α – glucosidase by peptide inhibitors might be an alternative against type 2 diabetes.
Zhipeng *et al.* (2012) [6] studied the anti-diabetic activity peptides from albumin against alpha amylase and reported that the peptides KLPGF had alpha amylase inhibitory activity. In this research work chicken intestine hydrolysate was used to study the antidiabetic bioactive properties in canine petfood using α-amylase inhibitory 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 10kDa 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 alpha amylase inhibitory assay.

Incorporation of Extracted Bioactive Peptides in Canine Pet Food

Based on the nutrient specifications and recommendations given by the AAFCO (2014) [1] 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 200g 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.

Anti-Diabetic Assay of Bioactive Peptides Incorporated Canine Pet Food at Different Incorporation Levels

Assay for α- amylase inhibitory activity

The alpha amylase inhibitory assay was performed on bioactive peptides incorporated canine pet food at different levels according to the method of Kim *et al.* (2004) [4]. Approximately 0.5 mg/ml stock concentration of canine pet food with bioactive peptides was dissolved in 1ml of 10% Dimethyl sulfoxide was used as stock solution for preparing required concentration in the form of liquid for alpha amylase inhibitory assay.

A. Preparation of reagents

1. Buffer solution (20 mM Sodium Phosphate with 6.7 mM Sodium Chloride, pH 6.9 at 20 °C) was prepared with solution containing 2.4 mg/ml of sodium phosphate, monobasic, and 0.39 mg/ml of sodium chloride, in distilled water and adjusted to pH 6.9 at 20 °C using 1 M NaOH/1 M HCl.
2. Starch solution [1.0% (w/v) Soluble Starch Solution] was prepared with concentration of 10 mg/ml solution using starch in Buffer: The solution was solubilized by boiling on a heating/stir plate for 15 minutes by mixing. The solution was allowed to cool at room temperature. Final volume was brought with addition of distilled water. The solution was mixed throughout the assay procedure.
3. Sodium hydroxide (NaOH) solution (2 M) was prepared with a concentration of 80 mg/ml using sodium hydroxide along with distilled water.
4. Potassium sodium tartrate tetrahydrate solution (5.3 M) was prepared with an concentration of 1,496 mg/ml in 2 M Sodium Hydroxide (NaOH) solution. The solids are dissolved by heating on a heating plate with mixing. Do not heat to a boil.
5. 3,5-Dinitrosalicylic acid solution (96 Mm) was prepared with a concentration of 21.9 mg/ml by using 3,5-Dinitrosalicylic acid in distilled water. The solids are dissolved by heating on a heating stir plate. Do not heat to a boil.
6. Colour Reagent solution was prepared to a volume of 100 ml by the addition 30 ml of warm (50–70 °C) distilled

water to an appropriate size amber bottle.

With mixing, slowly 20 ml of warm 5.3 M potassium sodium tartrate, tetrahydrate solution and 20 ml of warm 96 mM 3,5-Dinitrosalicylic acid solution was added. This solution is stable for 6 months at ambient temperature if protected from light.

7. α-Amylase Sample solution prepared by dissolving 0.3 grams of alpha amylase 10 units/ml in distilled water.

B. Protocol for alpha Amylase assay and IC_{50} (Inhibitory Concentration) value

1. 10µl of α- amylase solution was pre-mixed with 10µl of sample solution at different concentrations (in 10% DMSO).
2. Following incubation for 15 minutes, 500µl of 1% starch solution in sodium phosphate buffer (pH6.9) was added to start the reaction. The reaction was carried out at 37.5 °C for 5 minutes and terminated by addition of 600µl of the DNS reagent.
3. The reaction mixture was placed in a water bath at boiling point for 15 minutes and then cooled down to room temperature.
4. The α-amylase activity was determined at 540nm by a spectrophotometer.
5. Inhibitory activity was calculated by the following equation

$$\alpha\text{- amylase inhibitory activity } (\%) = \frac{A-B}{A} \times 100$$

Where: A was the optical density of reaction blank. The reaction blank mixture contained the same volume of the buffer solution instead of the sample; B was the optical density of the reaction in the presence of both α –amylase and peptide sample.

The effective concentration of sample required to inhibit α-amylase enzyme by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting 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

Alpha amylase inhibitory assay among different incorporation levels in canine pet food. The mean±SE values of IC_{50} value for anti-diabetic activity of bioactive peptide incorporated at different levels of 5%, 6% and 7% in canine pet food were 59.26±4.4 mg/ml, 364.28±13mg/ml and 163.57±5.04 mg/ml respectively as given in table 2.

These values were then compared with the IC_{50} value for anti-diabetic activity of canine pet food prior to addition of bioactive peptides. Which was taken as control is 1313.85±52.8. The test of significance revealed that there was a highly significant difference between the different levels of bioactive peptides incorporated canine pet food. The 5% inclusion level was found to have less IC_{50} value of 59.26±4.4 µg/ml and better alpha amylase inhibition activity.

Table 2: Mean±SE values of α - amylase inhibitory anti-diabetic assay among different incorporation levels of extracted bioactive peptides in canine pet food

Anti-diabetic activity	IC_{50} value
Pet food	1313.85±52.8
5% incorporation level in canine pet food	59.26^{a}±4.4
6% incorporation level in canine pet food	364.28^{b}±13
7% incorporation level in canine pet food	163.57^{c}±5.04

NS -Not Significant

* - Significant ($p<=.05$) difference

** - Highly significant ($P<0.01$) difference Means bearing different superscripts in the same row differ significantly

When compared with control pet food and other levels. The IC_{50} values of control pet food, six percent and seven per inclusion levels are 1313.85±52.8 µg/ml, 364.28±13 µg/ml and 163.57±5.04 µg/ml. it indicates that the potency of the extracted peptides was minimal when compared with standard and other levels. Anti-diabetic activity was more potent at 5% level of incorporations than the other levels.

Conclusion

Thus chicken intestine can be utilized for inclusion in pet food. It reveals that bioactive peptides obtained from chicken intestinal hydrolysate was having anti-diabetic activites in alpha amylase inhibitory 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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