Middle-East Journal of Scientific Research 11 (7): 881-886, 2012
ISSN 1990-9233

Production of Feather Protein Concentrate from Feathers by *In vitro* Enzymatic Treatment, its Biochemical Characterization and Antioxidant Nature

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Abstract: This study evaluated the effect of alkaline protease with NaOH pre-treatment on *in vitro* feather digestion. The biochemical and nutritional constituents like protein content, amino acids, water content, ash content and fat content of the feather protein concentrate (FPC) was evaluated. The antioxidant potential of the characterized FPC was also determined. Full solubilisation of feathers was achieved after pre-treatment with 0.3 M NaOH solution at 80° C, mechanical disintegration and enzyme hydrolysis at 55-60°C. Feather protein concentrate was obtained as a greyish powder with a mass of 19,100 g as the final product of *in vitro* feather digestion. FPC had a density of 0.332 g cm^{-3}. Its solubility in water, at pH 7.0, was 60-80%. The amino acid composition of FPC did not differ considerably from that of the feathers. Free radical-scavenging activity and reducing power showed the antioxidant potential of FPC. The obtained FPC is a source of soluble proteins, amino acids and other valuable products which could lead to the possibility of application in a large-scale production.

Key words: Alkaline Protease · Naoh · Feather Protein Concentrate · Amino Acid · Antioxidant

INTRODUCTION

Feathers are produced in large amounts as a byproduct at poultry processing plants, reaching millions of tons annually. Since feathers are almost pure keratin protein consisting of amino acids, feather wastes represent a potential alternative to expensive dietary ingredients for animal feedstuffs [1,2]. The protein shortage for food and feed leads us to look for a new protein sources from wastage products like feather wastes [3]. Feathers are significant source of protein for livestock because of their high protein content (>85% CP) [4-9].

Feathers contain large amounts of cystine, glycine, arginine and phenylalanine [10,26]. Raw feathers, however, are very poorly digested by non-ruminant animals because they contain a high proportion of keratin protein that has cystine disulfide bonds [11,12]. The indigestible structure of raw feather must be hydrolyzed to be used as a feed ingredient for non-ruminant species. Though keratin can be completely dissolved by reducing agents like copper sulphate, mercapto acetate, iodoacetic acid, amino, sodium sulphite, sodium tetrathionate [13-15] these methods are not suitable for the large scale application. In order to overcome these limitations, the use of microbial enzymes which improves the nutritional value of feather wastes has been implemented in recent years.

Several researchers have investigated chemical or enzymatic methods for the hydrolysis of feathers [10,16,17]. Steiner *et al*. [16] treated feathers with various concentrations of NaOH or H_3PO_4 and found they increased *in vitro* pepsin digestibility of feathers. Papdopoulos [10] treated broiler feathers with 0.2-0.6% NaOH or 0.2-0.6% maxatase. This study indicated that enzyme or NaOH treatment cleaved cystine disulfide bonds and improved feather solubility and susceptibility to digestive proteolytic enzymes. There are only a few studies that have evaluated the effect of NaOH or combining NaOH and enzymatic treatments for feather digestion. This study aimed to evaluate the effect of alkaline protease with NaOH pre-treatment on *in vitro* feather digestion. The biochemical and nutritional constituents like protein content, amino acids, water content, ash content and fat content was evaluated.

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Finally, the antioxidant potential of the characterized feather protein concentrate (FPC) was determined.

MATERIALS AND METHODS

Feather Material: White chicken feathers were collected from the local poultry farm near Chennai. They were washed thrice with distilled water and dried at 45° C for 48h. The feathers were stored at room temperature prior to experimental use.

Reagents: All the chemicals of analytical grade within the requirements were used. The alkaline proteases (Sigma Co., USA) from *Bacillus* sp. with an activity of 45,000 U g^{-1} were used for the feather hydrolysis.

Production of Feather Protein Concentrate (FPC): The feathers were processed in a glass reactor with a volume of 3 l with temperature control. The reactor was fed with 1 l of 0.3 M NaOH (pH 8, 80 °C). The Reactor temperature was increased to 180° C and 500 g of feather was added. The alkaline treatment was carried out at these conditions for 30 min at 60 rpm. After alkali treatment, the pH of the reaction mixture was adjusted to 8.5. It was followed by the addition of 1.25 g of alkaline protease. The temperature was adjusted to 55° C and maintained throughout the process. The feather hydrolysis was favored by stirring the reaction mixture for 2 h at 120 rpm. After feather hydrolysis, the pH was adjusted to 7 according to the protocol of Dalev [18]. Finally, the enzyme activity was stopped by raising the temperature to 95° C and kept for 15 min. This was done to avoid further hydrolysis reaction. After this step, the feather hydrolysate was concentrated to obtain feather protein concentrate.

Analytical Assays: Standard methods for analysis of foodstuffs were used for determination of protein, fat, minerals, water etc [19]. Amino acid analysis was carried out on an automatic analyzer, after hydrolysis of 2 mg of the sample with 4 ml 6N HCl for 24 h at 110°C in a sealed tube (cysteine and methionine were determined after oxidation of the sample using performic acid.

Antioxidant Activity: The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of the free radical scavenging activity of the extracts (AED and MED) by the method of Koleva *et al.* [20]. For each extract and standard, sample solutions of different concentrations (0.5-3.5 mg/ml) were prepared in methanol and added separately to an equal volume of 100 µM DPPH solution in methanol. The reaction mixture was kept at room temperature for 15 min. Then, the absorbance of the reaction mixture was recorded at 517 nm using a UV-visible spectrophotometer [Shimadzu UVPC-3200 (Kyoto, Japan)]. Gallic acid (GA) was used as standard. Free radical scavenging activity was calculated using the following formula:

$$\% \text{ of free radical scavenging activity} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

The extract concentration having 50% radical inhibition activity (IC_{50}) was calculated from the graph of the free radical scavenging activity (%) against the extract concentration. Three replicates were performed for each sample concentration to check the reproducibility of the experimental result and to get more accurate result. Results are represented as IC_{50} ± standard deviation.

Reducing Power Assay: The iron reducing ability of the FPC was analyzed by Yildirim *et al.* [21]. It was done by preparing different concentrations of FPC and then adding 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide to 1 ml sample of each hydrolysate. Then it was kept at 50° C for 30 min, followed by addition of 2.5 ml of 10% (w/v) trichloroacetic acid. After incubation the sample was centrifuged at 15,000×*g* for 10 min. the absorbance of the supernatant was done at 700 nm after incubation of the mixture containing 2.5 ml of the supernatant solution with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride for 10 min the sample with high reducing power was identified by increased absorbance.

RESULTS AND DISCUSSION

Enzymatic conversion of keratinous wastes constitutes a potential approach for their biodegradation and valorisation with respect to cost-effectiveness and environment protection [22]. Keratin is dissolved comparatively easily by means of reducing agents such as mercapto-acetate, alkylation with iodoacetic acid, copper sulfite, ammonia and sodium tetrathionate in the presence of 8 M urea, etc. [13-15]. These approaches are, however, unsuitable for a large-scale application. In a large-scale process the main requirement is the application of accessible and not toxic reagents to obtain a product with a high nutritive value. This study evaluated the effect of alkaline protease with NaOH pre-treatment on *in vitro* feather digestion.

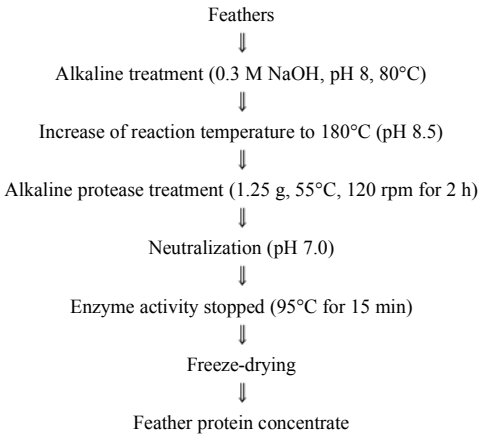

Fig. 1: Production of feather protein concentrate

The feathers were processed in a glass reactor with a working volume of 3 l supplied with a mechanical stirrer, which could be regulated between 60 and 180 rpm. The reactor was fed with 1 l of 0.3 M NaOH (pH 8, 80 °C). The Reactor temperature was increased to 180 °C and 500 g of feather was added. The alkaline treatment was carried out at these conditions for 30 min at 60 rpm. After alkali treatment, the pH of the reaction mixture was adjusted to 8.5. It was followed by the addition of 1.25 g of alkaline protease. The temperature was adjusted to 55° C and maintained throughout the process. The feather hydrolysis was favored by stirring the reaction mixture for 2 h at 120 rpm. After feather hydrolysis, the pH was adjusted to 7 according to the protocol of Dalev [18]. Finally, the enzyme activity was stopped by raising the temperature to 95 °C and kept for 15 min. This was done to avoid further hydrolysis reaction. At this stage of the process the reaction mixture was a thick and turbid solution. After spray-drying the product was a greyish powder with a mass of 19,100 g. This was 'Feather protein concentrate' (FPC). The scheme of the process is shown on fig. 1.

Characteristics of the FPC: The final product of feather processing was the feather protein concentrate (FPC). As a bulk material FPC was a powder with a greyish colour and a density of 0.332 g cm^{-3}. Its solubility in water, at pH 7.0, was 60-80%. The taste was salty but not unpleasant. The smell was specific. The data for the chemical characteristics are shown in table 1. As shown the difference in the data for feathers and FPC is negligible. According to the protein content, FPC can be added to the group of 'protein concentrates' of animal origin such as fishmeal (700 g kg^{-1} protein) blood meal (820 g kg^{-1}) etc. FPC considerably exceeded in protein content all protein concentrates of vegetable origin as soy bean grouts (480 g kg^{-1} protein content), sunflower grouts (390 g kg^{-1}) etc.

Table 1: Chemical composition of FPC and feather (g kg^{-1} to dry mass)

Constituent	FPC	Feather
Water	51	494
Crude protein	91	890
Fiber	6.4	-
Fat	13.2	14.3
Ash	85.2	62.1
Calcium	3.2	3.4
Phosphorus	0.9	1.1
Sodium	14.4	4.6
Chloride	20.7	7.6

Table 2: Amino acid composition of FPC and feather (g kg^{-1}protein)

Amino acid	FPC	Feather
Ala	55.6	58.9
Arg	70.1	67.7
Cys	42.4	44.7
Glu	102.8	101.5
Gly	76.9	76.5
His	13.7	14.1
Ile	51.5	49.1
Val	69.9	74.1
Leu	86.1	84.2
Lys	23.7	22.1
Phe	50.5	52.1
Pro	89.2	90.5
Ser	112.5	114.2
Thr	44.5	48.7
Tyr	24.5	24.2

The content of crude fiber was comparatively low so this would not be a limiting factor for application in a mixed feed for broilers where low fiber content is very important. Ash content is slightly higher than the native feathers, resulting from the neutralization with HCl and it could not be a limiting factor as salt is an indispensable component of broiler feeds. The other minerals in FPC are as much as in feathers. A great difference between feathers and FPC in chemical composition would not be expected. The same is true for amino acid composition as well (Table 2). The findings of the study are in accordance with Dalev [18] who reported similar findings in his study on FPC.

Table 2 shows the amino acid composition of FPC which did not considerably differ from that of the feathers. The main essential amino acid, which is a basis for balance of combined feed for broilers, is Lysine.

Table 3: Chemical score of FPC (%) and standard protein for essential amino acids (mg g^{-1})

Amino acid	Protein standard	Chemical score
Ile	40	128.1
Leu	70	123.2
Lys	55	43.1
Phe+Tyr	60	125.3
Thr	40	112.1
Val	50	140.5
Met+Cys	35	294.1

The feathers and FPC exceed considerably in Lysine (23.7 g kg^{-1} lysine content), all cereals such as maize, wheat and its content is very similar to that of green pea (18.0 g kg^{-1}), sunflower grouts (15.0 g kg^{-1}) and peanut grouts (16.0 g kg^{-1}). However, this is lower than in fishmeal (52.0 g kg^{-1}) and soy grouts (59.0 g kg^{-1}). Methionine is also an essential amino acid of great importance. Its content is three times higher than in cereal fodder, it is almost equal to that of soy grout but twice or three times lowers than in fishmeal. Nevertheless, the complete amino acid composition of FPC characterizes the product as a good source for preparation of mixed feed in combination with other protein products or with mixtures of synthetic amino acids.

Nutritional Characteristics of FPC: The high nutritional value of FPC can be expressed in percentage through the relation of the essential amino acids in the FPC protein and in the protein standard of FAO/WHO [23]. This relation has been named 'chemical score' (CS) or 'amino acid number' (AAN) and it is calculated through the formula:

$$CS=\frac{a}{b}\times 100$$

whereas *a* is quantity of the amino acid (in mg/g) in the protein investigated and *b* is the quantity of the same amino acid (in mg/g) in the protein standard. The theoretical model suggested by the General Committee of WHO experts is considered as a protein standard. The scale of the essential amino acids of the protein standard and the estimated values of CS of the essential amino acids in FPC are shown in table 3. Lysine excepted, the CS of all essential amino acids of FPC are above 100%. Remarkably high is the CS for the sulphur-containing amino acids. All this means that FPC could be used as a constituent of feeds, where a correction of sulphur content was necessary, as well as a constituent of all kinds of feeds.

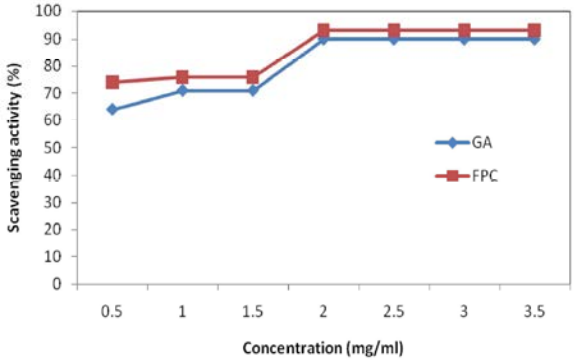

Fig. 2: Antioxidant activity of FPC.

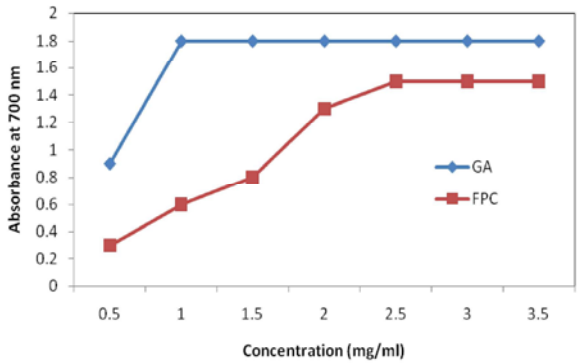

Fig. 3: Reducing power of FPC.

Antioxidant Activity and Reducing Power of FPC: DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance would be reduced [24]. The decrease in absorbance is taken as a measure for radical-scavenging activity. The DPPH radical-scavenging activity was investigated at different concentrations (0.5-3.5 mg/ml) of the FPH. The results presented in fig. 2 clearly show that the FPC exhibited an interesting radical scavenging activity with an IC_{50} value of 0.5 mg/ml.

It was worthy to note that the FPC, produced in this study, exhibited high DPPH free radical-scavenging activity which is comparable to that obtained from the findings of Fakhfakh *et al.* [22] using the strain *Bacillus pumilus* A1. The IC_{50} value of FPC (0.4 mg/ml) was lower than that of protein hydrolysate from smooth hound (0.6 mg/ml) using DPPH assay. The reducing power of the FPC was investigated at different concentrations and was to be concentration dependent. Its value increased with the higher FPC concentrations as was reported by Zhu *et al.* [25]. The reducing power results revealed that FPC, with high amino acid contents, could react with free radicals to form stable products (Fig. 3).

CONCLUSION

The proposed procedure for treatment of feather by a mixed protocol using alkali and enzymatic process is simple and economically viable. Therefore, it could serve as a basis in the development of a complex ecologically safe and efficient biotechnology for improved feather wastes utilization applicable to poultry-processing plants. The study showed the DPPH free radical-scavenging activity and reducing power showing the antioxidant potential of FPC. The obtained FPC is a source of soluble proteins, amino acids, enzymes and other valuable products and may be useful in agriculture for preparation of fertilizers and soil amendments, also in animal feeding as protein source.

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