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TABLE OF CONTENTS

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Primary quality assessment of keratin extracted from chicken feather waste as feed component

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Abstract

The present study suggests an effective method to solve the feather waste problem from poultry industries. Annually, the amount of feather waste is tendentiously increasing. This gives rise to an enormous amount of chicken feather waste, which is difficult to eliminate because of its resistance to the degradation. The main composition of the chicken feathers is a structural protein called keratin. This protein can compose up to 90% of feathers. The disulfide bridge (S−S) between cysteine in keratin causes it to possess high stability and water-insolubility. In case of improper feather discard, adverse impacts to the environment could possibly occur; e.g. pathogenic source creation and unwanted smells. Thus, the present work aims to seek for an appropriate method to utilize chicken feather waste. Chicken feathers were cleaned and degreased before being subjected to alkaline hydrolysis using a 2.5% NaOH solution (pH 13) at 70°C for 75 min. The extraction yield was 94.22 ± 0.72 %. The remaining debris was separated by centrifugation and was accounted for 5.78 ± 0.72 %. Consequently, the supernatant was collected and keratin was then precipitated by solid ammonium sulfate using 50% saturation and eventually harvested. Finally, the crude keratin was dried overnight at 80°C. The yields of crude keratin extract was 34.82 ± 1.84 %. Three samples of crude keratin extract were obtained and the quality of the crude samples was further analyzed by nitrogen content and in vitro digestibility to assess the possible use in feed component. By this method, feature waste was not only eliminated, but also valorized to obtain crude keratin, which can be further utilized in broad applications, especially in animal feed industry.

***Keywords*:** Keratin, waste valorization, alkaline hydrolysis, feed supplement

1. Introduction

The production of chicken meat worldwide has been constantly grown for over a decade. In Thailand, the production has obviously increased from 994.32 million broilers in 2011 to 1055.13, 1103.32, 1209.52 and 1310.58 million broilers in years 2012, 2013, 2014 and 2015 respectively (source: Office of Agricultural Economics). This leads to an enormous waste stream of feathers. Presently, the chicken feather waste derived from poultry industries in Chiang Mai and the neighboring provinces is currently managed under the two following practices. Firstly, feather waste is directly dried by sunlight and milled to an appropriate size and eventually used as animal feed. Secondly, feather waste is disposal as landfill. The former method adds only a small economic value to feather waste, whereas the latter can cause germ-breeding sources in case of unsanitary handling. Furthermore, land filling remarkably increases operating cost, since it requires wide areas and surface preparation expenses. These give rise to the search for an alternative method to manage the chicken feather waste and simultaneously to promote a full extension of chicken feather utility.

Feathers are mainly composed of a protein called keratin (Schrooyen *et al.*, 2001), a structural protein found in nature. Keratins are a component of hair, fur, feathers, horn and nail etc. (Sinkiewicz *et al.*, 2017). Their amino acid constituents, particularly cysteine make keratins distinct from other structural proteins such as collagen, silk and fibril (Yin *et al.*, 2007). The disulfide bonds (S−S) between cysteine, as well as the occurrence of a huge amount of nonpolar amino acids, give keratins a high stability and provide structural strength (Schrooyen *et al.*, 2001). On the one hand, the functions of keratins including toughness, water−insolubility and mechanical strength, are desirable in several applications. On the other hand, they cause the difficulty in utilizing keratins. An extraction of feather keratin is thus based on the breakage of disulfide bonds in keratin molecules (Nagai and Nishikawa, 1970). Therefore, the possibility to create benefit from keratins can be done by studying the proper methods to make them water soluble (Staron *et al.*, 2014); (Zhang *et al.*, 2013).

An interest in the utilization of proteins as a renewable resource has recently been increasing (Schrooyen et al., 2001). Chicken feather keratins are one promising candidates. Evidently, several methods on keratin extraction have been proposed. Yin et al. (2007) studied the use of boiling water at superheated state to hydrolyze keratin into short chain oligopeptides containing sequences of approximately 40 amino acids. They concluded that these oligopeptides can be used as significant building blocks for novel polymer synthesis.

An extraction method based on chemical hydrolysis by acid, alkaline and catalysts as well as enzymatic hydrolysis have been studied. For chemical hydrolysis, high temperature and pressure should be applied to the reaction, whereas an enzymatic hydrolysis is costly and time−consuming (Staron *et al.*, 2014). Zhang *et al.* (2013) found that acid hydrolysis more efficiently destroyed keratin structures and made them more water soluble. However, this method may lead to the loss of some amino acids such as tryptophan. In contrary, Coward−Kelly *et al.* (2006) proved that the reaction rate of alkali hydrolysis was slower than acid hydrolysis, but the loss of amino acid was less. Moreover, they found the

effectiveness of hydrolysis reaction to be dependent on pH value of the system, reaction temperature and the concentrations of alkaline and acid used.

Apart from acid/base hydrolysis, keratin can be extracted using reducing agents. Evidently, Sinkiewicz *et al.* (2017) prepared soluble keratin by the thermo−chemical treatment with 2−mercaptoethanol, dithiothreitol and sodium m−bisulphite. After the insoluble material was separated, the filtrate obtained was dialysed in distilled water using dialysis membrane (MWCO 3500−5000 Da). The authors illustrated that when adjusting the reaction condition at 50°C for 2 h the first two reducing agents gave the keratin yields at 84 and 82%, respectively. In case of using sodium m−bisulphite, the same yield could be obtained with an additionally increased 1−h extraction time.

Mokrejs et al. (2010) investigated the extraction of keratin via a two−stage alkaline−enzymatic hydrolysis. For the first hydrolysis stage, feathers were stirred with 0.1−0.3% KOH water solution at 70°C for 24 h. After pH adjustment to 9, the mixture was subjected to the second hydrolysis stage by adding 1−5% proteinase. Approximately 91% of feathers were degraded and the hydrolysate could be further utilized in packaging application.

A suitable keratin extraction process is in accordance with the purpose of further application. Evidently, hydrolysis process reducing or protecting amino acid loss is an obvious objective in using chicken feather in feed production (Coward−Kelly *et al.*, 2006) whereas the improvement of both physical and chemical properties of keratin to obtain bio−building block is an aim of biomaterial research (Rouse and Van Dyke, 2010). This study presents the method of keratin extraction by solubilizing chicken feather with 2.5% NaOH at 70°C for 75 min. The crude keratin powder was subjected to total nitrogen and amino nitrogen analyses as well as in vitro digestibility test in order to screen for the potential of keratin in animal feed application, with a special attention to domestic pets.

2. Materials and Methods

2.1 Keratin extraction

Fresh chicken feathers were collected from the poultry industry in Lamphun and Chiang Mai provinces in the northern part of Thailand. Twenty grams of feathers were soaked in 500 mL of water containing 0.3% (W/V) of sodium carbonate (EMSURE®, Darmstadt, Germany) and 0.1% (W/V) of triton X−100 (Calbiochem®, Darmstadt, Germany) at 70°C for
1 h. The sample was then washed twice with hot water (95°C) and washed for the last time with lukewarm water (40°C). The chicken feathers were then squeezed to remove the remaining water and dried before being cut into small pieces. Later, dried cut chicken feathers were immersed in hexane (EMSURE®, Darmstadt, Germany) at room temperature with shaking overnight to remove grease. The feathers were filtered and left for drying under fume hood whereas grease was separated from solvent using a rotary evaporator (Buchi, Flawil, Switzerland). The solvent was kept for reuse.

The keratins were extracted from degreased sample by the alkaline hydrolysis method described by Sinkiewicz *et al.* (2017) with a minor modification. Briefly, defatted feathers were mixed with 2.5% NaOH (EMSURE®, Darmstadt, Germany) at the ratio of 1:20 g/mL at 70°C for 75 min using a magnetic stir bar.

Consequently, the reaction mixture was centrifuged (Hettich, Tuttlingen, Germany) at 4000 rpm for 20 min to separate the insoluble debris. The supernatant was filtered and then collected. Solid ammonium sulfate (Calbiochem®, Darmstadt, Germany) was subsequently added to the filtrate to 50% saturation. Keratin was finally collected and freeze dried. Alternatively, keratin could be dried overnight at 80°C in the hot air oven (Memmert, Schwabach, Germany) to obtain the constant dry weight.

2.2 Quality assessment of crude keratin

2.2.1 Nitrogen content analysis

Protein content of keratin extract was determined by the Kjeldahl method (Kjeldahl, 1883); (Patsanguan *et al.*, 2014). Briefly, the dried crude keratin sample was digested with 98% H2SO4 solution (Labscan, Bangkok, Thailand) at high temperature (300−400°C) until the liquor was clarified. The clear sample solution was then distilled with 32% NaOH solution (pH 14). The amount of nitrogen in a sample was measured via titration. Free alpha amino nitrogen (FAN) was analyzed using ninhydrin method according to Lie (1973). In short, 1 g of crude keratin was mixed with 100 mL of distilled water to obtain keratin suspension sample. Later, 2 mL of sample was mixed with 1 mL of color reagent containing ninhydrin 5 g/L (Ajax Finechem, New South Wales, Australia) and heated for 16 min in a constantly boiling water bath. After the sample was cooled down for 20 min, the diluted solution (prepared by dissolving 2 g of KIO3 in 600 mL of distilled water and adding 400 mL of 96% ethanol) was then transferred to the tube and mixed thoroughly. Finally, the formation of complex compound was monitored at 570 nm by spectrophotometer (Drawell, Shanghai, China). The glycine solution was used as a standard. The value was expressed as mg FAN/g dry weight, when content of FAN was given by following equation:

Mg/l of FAN = (OD. of test solution / mean OD. of standard) x 2 x dilution

2.2.2 In vitro digestibility test

The method used in this study was partially adapted from Biagi et al. (2016). The calculation of % digestibility was conducted according to the following steps including gastric digestion simulation, small intestine simulation and collection of an undigested fraction.

Gastric digestion simulation: pepsin−HCl solution was prepared by dissolved 2 g of pepsin (Sigma−Aldrich, St. Louis, USA) in 0.075 N HCl (Labscan, Bangkok, Thailand). To start the test, 1 g of dry keratin was added to 40 mL of pepsin−HCl solution. The mixture was then shaking in water bath at 39°C for 2 h.

Small intestine digestion stimulation: pancreatin solution was prepared by mixing 2 g of pancreatin (Sigma Aldrich, St. Louis, USA) in phosphate buffer. To continue the test, the mixture from gastric digestion step was adjusted to reach the pH of 7.5 with NaOH prior to the addition of 40 mL pancreatin solution. Consequently, the bottles were placed in a shaking water bath at 39°C for 4 h.

Collection of an undigested fraction: the mixture was centrifuged at 400 rpm for 5 min at 4°C in order to separate an undigested fraction. The supernatant was decanted and the fraction was then washed twice with distilled water. The residue

was dried at 85°C until the constant weight was obtained. The % digestibility was calculated using the following equation:

$$\% \text{ digestibility} = 100 - [(\text{residue weight} \times 100) / \text{sample weight}]$$

2.2.3 Analysis of amino acids

Amino acid profile was sent to analyze by Central Laboratory, Faculty for Agriculture, Chiang Mai University via ARACUS amino acid analyzer, Membrapure, Germany.

3. Results and Discussion

3.1 Keratin extraction

The fresh chicken feathers were prepared prior to alkaline hydrolysis. After cleaning and drying (fig 1A), feathers were degreased in hexane overnight at ambient temperature. The solvent was removed from the feathers in the hood to obtain defatted feathers (fig 1B). The amount of grease removed was accounted for 4.52 ± 0.44%.

Fig 1 The preparation of chicken feathers prior to the alkaline hydrolysis: (A) chicken feathers after cleaning, (B) defatted chicken feathers.

Defatted chicken feathers were cut into small pieces and subjected to alkaline hydrolysis (fig 2A). Feathers were constantly dissolved (fig 2B) and finally appeared as homogenous thick slurry. An undissolved debris was separated from the slurry by centrifugation (4000 rpm for 20 min). Up to 94.22 ± 0.72 % of the feathers were soluble in 2.5% NaOH solution at 70°C for 75 min.

Fig 2 Keratin extraction via alkaline hydrolysis: (A) feathers were mixed with 2.5% NaOH solution (pH 13) and equipped on hot plate, (B): feathers were constantly solubilized over time.

In comparison to other related studies, a two−stage alkaline−enzymatic hydrolysis was reported to reach a maximum degradation of 90.83% under an optimum environment (Mokrejs *et al.,* 2010). Similarly, Nagai and Nishikawa (1970) were able to extract 90% of keratin exploiting 40 mL of 0.1 N NaOH to 1 g of dry feather at 90°C for 15 min. Therefore, the method exploited in this study found to be effective to make use of chicken feathers, since a higher percentage of feathers was dissolved. This means about 95% of the chicken feathers were further processed to obtain keratin, whereas approximately 5% of an undissolved residue should be rinsed with water to obtain neutral pH (pH ≈ 7) and, eventually, directly released to the environment by means of composting. At this stage, no fresh feathers were left after the dissolving process.

The supernatant obtained from alkaline hydrolysis was collected for keratin precipitation via solid ammonium sulfate. The keratins were salted out from the solution and further separated from the solution by centrifugation (4000 rpm for 20 min). Later, the keratins were separated from the liquid phase via filtration and dried in the hot air oven at 80°C until the constant dry weight could be obtained (fig 3A). Alternatively, the crude keratins were lyophilized to obtain a constant dry weight within a shorter period. The keratin yield was 34.82 ± 1.84%.

Fig 3 The solubilized keratins were recovered by ammonium sulfate precipitation: (A) crude keratins were dried in hot oven until the constant weight was reached, (B) the end product after an extraction process.

Instead of using a salting out process, Sinkiewicz *et al.* (2017) harvested keratin via dialysis in distilled water for 72 h. The dialysis method is salt−free, which reduces the step of separating salt from the liquid phase before discard. However, this process is time−consuming and requires high expense, whereas the ammonium sulfate employed in this study gives a short operating time with an affordable price. Nevertheless, salt separation cost should be taken into account and included in keratin extraction cost.

3.2 Quality assessment of crude keratin

3.2.1 Nitrogen content analysis

The dried crude keratin sample was analyzed for the total Kjeldahl nitrogen (TKN). The nitrogen was 61.63% (SD ± 0). The amount of alpha-amino nitrogen was measured according to ninhydrin method. The sample contained 5.34 ± 0.01 mg FAN/g dry crude keratin.

3.2.2 *In vitro* digestibility

To verify the possible use of crude keratin extract as feed component, the keratin sample was tested for the digestibility. Because of the moral issue, the use of experimental animals has obviously become a great concern. Additionally, the European legislation for the protection of companion animals is very restricted for the purpose of experiment. Thus an alternative reliable *in vitro* method was conducted in this study. The tests were undertaken in triplicate and the digestibility was accounted for 98.72 ± 0.51 %. The results are illustrated in table 1.

The results illustrate that the crude keratin extracted via alkaline hydrolysis was almost completely digested by the digestive system of domestic pets such as dogs. This opens up the possibility to utilize feather keratins as a feed component, such as a feed supplement as well as a feed additive depending on the exact properties of the feather keratins. Thereby, the properties of feather keratins require further studies to extend the full usage of feather keratins.

Table 1 The results of *in vitro* digestibility test for dogs according to method suggested by Biagi *et al.* (2016)

Experiment no.	Sample initial weight (g)	residue weight (g)	% digestibility
1	1.00	0.0200	98
2	1.00	0.0089	99.11
3	1.00	0.0035	99.05

6.2.3 Analysis of amino acids

The crude keratin extract was analyzed for an amino acids profile using the amino acid analyzer. The result showed that all essential amino acids, including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, were found in the keratin sample. The content of each amino acid are illustrated in table 2.

Since essential amino acids are of a type that the body cannot produce. They are constantly metabolized over time, and must be regularly restored via diet. Therefore, the feather keratin sample extracted by alkaline hydrolysis from this study was found to be suitable for the purpose of feed and dietary supplement application because it contains all kind of essential amino acids, despite the loss of amino acid tryptophan in keratin extract via acid hydrolysis according to Zhang *et al*. (2013). However, the potential use of feather keratin extract as a feed component should further be verified with a special attention to chicken derived protein allergies in domestic pets. This aspect faces ongoing research to ensure the safety of utilizing chicken feather waste in animal feed.

Table 2 The list of amino acids and their content (mg/100g sample) found in the crude feather keratin extract.

Amino acids found in crude feather keratin extract	Amount (mg/100g)
Alanine	1,117.86
Arginine	1,100.07
Aspartic acid	2,011.14
L−Cystine	280.88
Glutamic acid	2,711.24
Glycine	3,235.70
Histidine	299.30
Hydroxylysine	62.90
Isoleucine	495.17
Leucine	2,395.49
Lysine	307.36
Methionine	720.05
Phenylalanine	1,648.60
Proline	3,289.55
Serine	1,981.73
Threonine	352.74
Tryptophan	155.73
Tyrosine	901.32
Valine	938.08

4. Conclusion

This study suggests an eco−friendly and practical method to manage chicken feather waste derived from poultry production. Nearly 95% of chicken feather was dissolved by alkaline hydrolysis and extracted for crude keratins, whereas circa 5% of the rest could be decomposed and directly released in the environment. Thus, the chicken feather waste was completely removed from the environment via this proposed practice. According to the digestibility and amino acid analysis results, the crude keratins show to have potential as component of animal feed, especially in domestic pets. However, further study is required to promote the full extent of keratin in feed industry. Although the feather waste was valorized in this study, the technique introduced to the system also needs to be optimized to establish a whole zero−waste process including chemicals and materials used throughout the process.

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