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Original article

Keratin hydrolysate from chicken feather using alkaline-enzyme hydrolysis

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Abstract

***Background**:* Chicken feathers are a major byproduct of the poultry industry, containing 91% keratin protein. The protein's structure, with its hydrophobic interactions and disulfide bonds, makes it difficult to break down using conventional methods. However, these structural properties make chicken feather keratin a valuable resource for product development.

***Objectives**:* This study aims to develop and evaluate a combined alkaline-enzyme hydrolysis method for keratin extraction from chicken feathers. The research examines molecular weight distribution and ionic characteristics of both soluble (S-KAH) and precipitate (P-KAH) keratin fractions.

***Methods**:* The alkaline-enzyme hydrolysis process used 0.5 M NaOH with 1% alcalase at 60°C for 22 hours. Subsequent fractionation at pH 4.5 produced soluble (S-KAH) and precipitate (P-KAH) fractions. Characterization involved ultrafiltration using 3 and 10 kDa membranes and ion-exchange chromatography.

***Results**:* The combined alkaline-enzyme hydrolysis method yielding 87% total keratin. Analysis revealed two distinct fractions: soluble keratin hydrolysate (S-KAH) constituted 72% of the yield, while precipitate keratin hydrolysate (P-KAH) comprised 17%. Ultrafiltration analysis showed that S-KAH predominantly contained peptide fragments under 3 kDa (58%), whereas P-KAH exhibited primarily larger fragments exceeding 10 kDa (81%). Ion-exchange chromatographic analysis at pH 7.0 revealed that both S-KAH and P-KAH displayed anionic characteristics with neutral peptide components.

***Conclusion**:* The alkaline-enzyme hydrolysis method demonstrated high efficiency in keratin extraction from chicken feathers, yielding high extraction rates and distinct protein fractions characterized by specific molecular weights and ionic characteristics. The results indicate potential for large-scale industrial application.

***Keywords**:* Alkaline-enzyme hydrolysis, chicken feather, keratin hydrolysate,

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Keratin biomass is one of the most structurally complex biological materials in nature. It is the primary component in hair, nails, tortoise shells, horns, beaks, claws, and feathers. Keratin is the third most abundant biopolymer in nature, after cellulose and chitin. [1] Chicken feathers, a significant byproduct of poultry processing, contain approximately 91% keratin, 8% moisture, and 1% lipid, representing 5-7% of the chicken body weight. [2] The extraction of keratin from chicken feathers provides a sustainable solution for managing industrial waste. The protein's complex structure, characterized by hydrophobic interactions and disulfide bonds, presents significant challenges for degradation processes. Unlike other natural polymers such as collagen, chitosan, and starch, keratin shows high resistance to water solubility and enzymatic degradation, requiring intensive extraction methods.[3] Alternative extraction methods incorporating environmentally sustainable and energy-efficient approaches have emerged, particularly the use of deep eutectic solvents for keratin dissolution.[4] Chemical treatments with NaOH, urea, or Na_2S can extract keratin through proteins dissolution and disulfide bond reduction. Key extraction parameters- include solvent ratio, concentration, pH, temperature, and reaction time [5] are followed by acid precipitation of keratin. [6]

The proteolysis of keratin proteins yields novel bioactive peptides with properties distinct from the parent protein structure. These peptides demonstrate potential for product development through their functional characteristics, including antioxidant and antimicrobial activities that enhance product shelf life, as well as water retention, emulsification, and solubility properties that improve textural attributes. [7] The use of exogenous enzymes for enzymatic hydrolysis is an established method for generating bioactive peptide from keratin proteins. [8] The selection of appropriate enzymes offers multiple advantages, including specific peptide bond cleavage, reproducible results, and predictable peptide generation patterns. Alcalase demonstrates stability in organic media and alkaline conditions, facilitating peptide bonds cleavage and improving antioxidant properties of resulting peptides. [9] Alternative approaches involve fermentation with keratin-degrading microorganisms, such as *Bacillus cereus* L10, with applications in hair treatment. [10] However, there is currently no established production process of keratin that effectively combines alcalase enzyme hydrolysis with size separation through pH precipitation at an industrial level. This study aimed to extract and characterize keratin from chicken feathers using alkaline-enzyme hydrolysis. The research focused on analyzing the molecular weight distribution and ionic properties of both soluble (S-KAH) and precipitated (P-KAH) keratin fractions through ultrafiltration and ion-exchange chromatography.

Materials and methods

Chicken feather powder was provided by Living Soil Thailand company, Chiang Mai province. Alcalase 2.4 L from *Bacillus licheniformis* (2.4 AU/mL) was purchased from Novozymes Co., Ltd. Other disposable materials and chemicals were purchased from local distributors.

Enzyme-alkaline hydrolysis of chicken feathers by alcalase

Prepare 200 mL of extracted solution with 7.2 g of feather powder per 100 mL in 0.5 M NaOH using a 500 mL Duran laboratory bottle. Stir at 150 rpm for 6 h with magnetic stirrer (Fisher Scientific: Isotemp) and maintain the temperature at 60°C. Adjusted the reaction to pH 11 with 1 M HCl. Add 1% alcalase (v/w; volume by weight of chicken feather) to the solution and shaken 150 rpm at 60°C for 16 h using incubator shaker (ZWY-240). Stop the reaction by boiling for 10 min. Cool to room temperature and centrifuge 4500 × g for 10 min. Collect the supernatant and acidify it to pH 4.5 by adding 1 M HCl. Refrigerate at 16°C for 16 h, followed by centrifuge at 9,200 × g at 4°C for 10 min. Collect the clear supernatant and adjust to pH 7 with 0.5 M NaOH, then store at 40°C. The precipitate was washed twice with distilled water, adjusted to pH 7.0 with 1 M NaOH to dissolve, and freeze-dried. The soluble fraction is called soluble fraction (S-KAH) while the precipitate from pH precipitation is called precipitation fraction (P-KAH). Calculate the percent yield by dividing the weight of the freeze-dried product by the initial chicken feather weight and multiplying by 100. Measurements were conducted in triplicate, with results expressed as mean values.

Molecular weight distribution

Each of the keratin fraction (S-KAH and P-KAH) was separated by using an ultrafiltration membrane with molecular weight cut-off (MWCO) size of 3 kDa and 10 kDa (Sartorius Co., Goettingen, Germany).

Each fraction was collected and freeze-dried to calculate % fraction by dividing the weight of the freeze-dried product from each fraction by total freeze-dried weight and multiplying by 100.

Ion-exchange chromatography

S-KAH and P-KAH samples were analyzed using ÄKTA go fast protein liquid chromatography (FPLC) system. Protein solutions (5 mg/mL) were dissolved in 20 mM Tris-HCl buffer (pH 7.0) and loaded onto either HiTrap Capto Q (anion exchanger) or HiTrap Capto S (cation exchanger) columns. The flow rate was maintained at 0.4 mL/min with system pressure not exceeding 0.5 MPa. Column equilibration was performed with 5 column volumes (CV) of 20 mM Tris-HCl buffer (pH 7.0). Sample injection volume was 0.5 mL. Unbound proteins were removed with 5 CV of equilibration buffer. Protein elution was conducted using a linear gradient of 0 1 M NaCl in 20 mM Tris-HCl buffer (pH 7.0) over 10 CV. Post-elution column washing utilized 5 CV of 1 M NaCl in equilibration buffer, followed by 5 CV of equilibration buffer. Protein distribution was analyzed by calculating % peak area.

Results

Physicochemical characteristics of keratin hydrolysates

The alkaline-enzyme hydrolysis process produced a dark brown solution when combining 0.5 M NaOH with alcalase. Chicken feathers were completely dissolved with minimal precipitate formation. Adjusting the keratin solution to pH 4.5 resulted in two distinct layers (**Figure 1A**). After centrifugation and freeze-drying, two fractions were obtained: a soluble fraction (S-KAH) yielding 5.2 0.2 g dry weight (72% yield) (**Figure 1B**) and a precipitate protein fraction (P-KAH) yielding 1.2 0.1 g dry weight (17% yield) (**Figure 1C**) from the initial 7.2 g of raw feather material.

Molecular weight distribution

Molecular weight analysis showed that alcalase hydrolysis yielded 52% of peptides with sizes under 3 kDa. The soluble fraction (S-KAH) contained predominantly peptides below 10 kDa (96%), while the pH-precipitated fraction (P-KAH) consisted mainly of larger protein components above 10 kDa (80.71%) (**Table 1**).

Figure 1. Demonstrates the pH precipitation at 4.5. Following refrigeration, protein precipitation reached completion (**A**). The freeze-drying process yielded yellow protein powder samples from both the solution (S-KAH) (**B**) and precipitate (P-KAH) fractions (**C**).

Table 1. Molecular weight distribution of keratin hydrolysate fractions obtained through ultrafiltration. KAH represents the total keratin hydrolysate, while S-KAH and P-KAH represent the soluble and precipitate fractions separated at pH 4.5, respectively.

Ultrafiltration Peptide sizes	KAH	% Fraction KAH soluble (S-KAH)	KAH Precipitation (P-KAH)
> 10 kDa	16.18	3.25	80.71
3-10 kDa	31.76	38.31	9.50
< 3 kDa	52.06	58.43	9.79

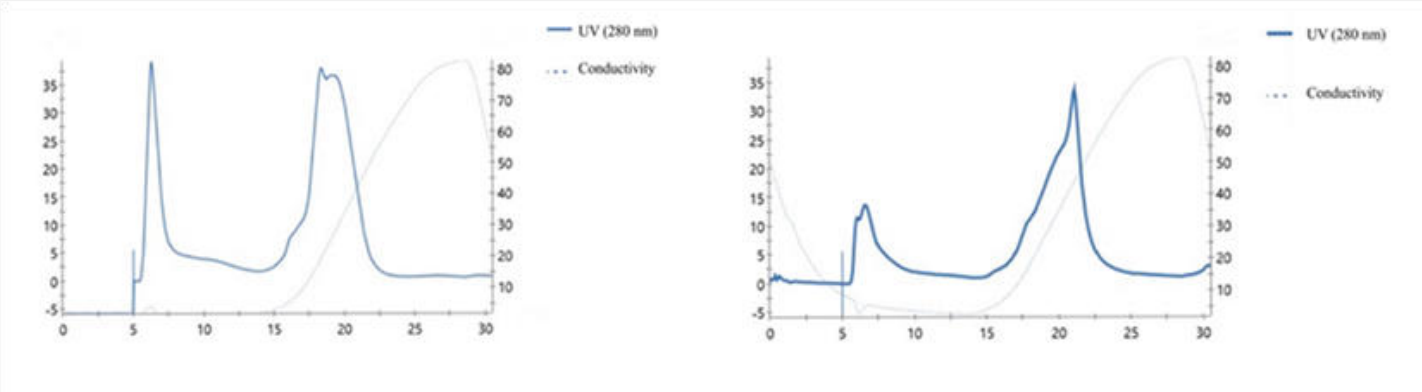

Figure 2. Illustrates the ion exchange chromatographic analysis using a HiTrap Capto Q column. The chromatograms show protein absorbance at 280 nm (blue line) and salt gradient concentration from 0 to 1 M NaCl (dotted line). The analysis reveals distinct elution profiles for S-KAH (**A**) and P-KAH (**B**) fractions. At pH 7.0, both fractions show significant binding affinity to the anion exchange resin, indicating predominantly negative charge characteristics.

Ion exchange chromatography

Ion exchange chromatographic analysis revealed distinct peptide distributions in both fractions at pH 7.0. The S-KAH fraction showed 73% protein binding capacity with the HiTrap Capto Q anion exchange column but no binding with the HiTrap Capto S cation exchange column, indicating 27% neutral peptides and 73% negatively charged peptides. The P-KAH fraction showed similar characteristics with 78% protein binding to the anion exchange column and no binding to the cation exchange column, representing 22% neutral peptides and 78% negatively charged peptides. These results indicated that P-KAH contained a higher proportion of negatively charged peptides compared to S-KAH at pH 7.0.

Discussion

Several factors influence the extraction yield, including keratin-to-solution ratio, temperature, and NaOH concentration. Previous research demonstrated that utilizing 1 M NaOH for 32 h at room temperature produced a 41% yield. [11] Subsequent studies revealed that adjusting conditions to 0.445 M NaOH at 87°C for 111 min increased the keratin hydrolysate yield to 68.3%. [5] The present study achieved nearly complete feather dissolution using 0.5 M NaOH at 60°C for 6 h. The application of urea and L-cysteine at 30°C for 1 h yields approximately 90% keratin extraction through the disruption of hydrophobic forces and disulfide bonds. This method minimizes the degradation of tryptophan, lysine, and methionine typically observed at elevated temperatures. However, the process necessitates an additional solution exchange step involving three days of dialysis with distilled water. [12] An alternative approach utilizing Ionic liquids (IL) with Deep Eutectic Solvents demonstrates potential applications in polymer processing due to their reported preservation of peptide structure. This method requires processing at 100°C for 6 h. [4] The enzymatic digestion of keratin using alcalase demonstrates optimal efficiency at pH 11.0 and 60°C, facilitating pH adjustment through HCl addition to the keratin solution in 0.5 M NaOH. Research demonstrates alcalase's superior performance in keratin digestion compared to alternative enzymes such as papain and neutrase, achieving 97% solubility within 2 h. Analysis reveals that over 85% of the resulting hydrolysates comprise peptides below 2 kDa. The enzyme exhibits selective specificity toward hydrophobic amino acids (Met, Val, Leu, and Ala) and hydrophilic amino acids (His and Gly) at the C-terminal. [9] The present study's 6-h digestion yielded approximately 52% of peptides below 3 kDa. Optimal keratin precipitation occurs at pH 4.5 using hydrochloric acid, corresponding to keratin's isoelectric point range of 4.9–5.4. [11] Precipitation of

keratin for bioplastic applications is performed at pH 4.7.[13] Analysis indicates predominant precipitation of peptides exceeding 10 kDa, enabling size-based fractionation through precipitation methods. The alkaline extraction process has several limitations, including formation of undesirable byproducts like lysinoalanine compounds, environmental concerns due to NaOH usage, and high-water consumption during the precipitation phase. [14] Characterization of keratin hydrolysate at pH 7.0 demonstrated distinct peptide distributions, with neutral and negatively charged peptides present in a 1:3 ratio. The precipitated fraction contained elevated levels of negatively charged peptides, exceeding 5% of total content. Further applications focused on hair treatment protocols utilizing the soluble KAH fraction, specifically employing neutral, low molecular weight peptide components.

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Conflict of interest statement

All authors have completed and submitted the International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for non-commercial purposes from the corresponding author on reasonable request.

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