Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

EXTRACTION OF β-KERATIN FROM POULTRY FEATHER WASTE USING SODIUM METABISULFITE AND SODIUM DODECYL SULFATE

(Pengekstrakan β-Keratin dari Sisa Bulu Ayam Menggunakan Natrium Metabisulfit dan Natrium Dodesil Sulfat)

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Received: 14 September 2023; Accepted: 18 November 2023; Published: 28 February 2024

Abstract

Environmental contamination due to the disposal of poultry feather waste requires careful attention and management. As poultry feathers are a rich source of keratin protein, this study aims to determine the most efficient reducing agents for extracting β-keratin from chicken and duck feather waste as well as their optimal concentrations. For this purpose, sodium metabisulfite ($Na_2S_2O_5$) and sodium dodecyl sulfate ($NaC_{12}H_{25}SO_4$) at different concentrations, namely 0.2 M, 0.5 M, and 1 M, were utilized as the reducing agents. The results showed that the optimal concentration of reducing agents for extracting β-keratin from both chicken and duck feather waste was 0.2 M. Sodium metabisulfite (SMB) was proven to be the most effective reducing agent for chicken feather waste, with a yield of 76.9%. For duck feather waste, on the other hand, the use of sodium dodecyl sulfate (SDS) resulted in β-keratin extraction of 39.8%.

Keywords: β-keratin, feather waste, chicken feathers, duck feathers, sodium dodecyl sulfate

Abstrak

Pencemaran alam sekitar yang disebabkan oleh sisa bulu unggas memerlukan perhatian dan ditangani secara serius. Bulu unggas dapat dimanfaatkan sebagai salah satu sumber protein keratin. Kajian ini bertujuan untuk menentukan agen penurunan yang paling berkesan dan kepekatan optimum untuk pengekstrakan β-keratin dari sisa bulu unggas (bulu ayam dan bulu itik). Natrium metabisulfit ($Na_2S_2O_5$) (SMB) dan natrium dodesil sulfat ($NaC_{12}H_{25}SO_4$) (SDS) dengan pelbagai kepekatan 0.2 M, 0.5 M, dan 1 M telah digunakan sebagai agen penurunan. Kepekatan yang berkesan untuk pengekstrakan β-keratin dari sisa bulu ayan dan itik adalah 0.2 M. Agen penurunan yang berkesan untuk mengekstrak β-keratin daripada sisa bulu ayam adalah SMB menghasilkan sebanyak 76.9%. Manakala penggunaan SDS dalam sisa bulu itik menghasilkan 39.8% β-keratin.

Kata kunci: β-keratin, sisa bulu, bulu ayam, bulu itik, natrium dodesil sulfat

Introduction

As a global problem that requires careful attention and management, environmental pollution is caused by various contaminants, one of which is poultry feather waste [1]. It is estimated that around 8.5 billion tons of feathers are produced by the poultry processing industry

as waste every year throughout the world [2]. This waste is usually disposed of through incineration and landfilling. Human population growth, increasing income, and changes in lifestyle and diet have caused a rapid increase in livestock production [3]. However, the growth in poultry production also contributes to environmental pollution, especially because the feathers are underutilized in various applications.

Poultry feathers are rich in keratin protein which can be used in various fields, including green chemistry, food science, pharmaceutical industry, biomedicine, hair cosmetics, and composite materials [4]. Keratin is the third most abundant polymer after cellulose and chitin. Due to its unique and non-toxic biodegradability and biocompatibility properties, keratin can be modified and developed into various forms, such as gels, films, beads, and nano/microparticles. Feather keratin is a small and uniform protein with a molar mass of 10-36 kDa. The structure of this keratin is stabilized by various covalent (e.g., disulfide bonds) and non-covalent interactions (e.g., electrostatic forces, hydrogen bonds, hydrophobic forces) [5]. Meanwhile, the high strength and stiffness of keratin are due to the high proportion of cysteine residues in the polypeptide backbone, which are held together by disulfide bonds [4].

The average poultry feather consists of 32.2% α-helix, 53.6% β-sheet, and 14.2% random coils that form turns [3]. Chicken feathers contain more than 85% β-keratin, while duck feathers are an excellent source of β-keratin-based protein comprising around 90% β-keratin [6]. β-keratin can be extracted through hydrolysis, reduction, and oxidation [7]. Among the three, the reduction process is more commonly used for β-keratin extraction owing to its high efficiency. Reducing agents decrease the stability of β-keratin fibers by disassociating disulfide bonds and hydrogen bonds as well as allowing protease access to the polypeptide backbone to dissolve proteins into solution. In the presence of reducing agents, the solubility of β-keratin increases at high temperature [4].

Numerous prior studies have demonstrated the successful extraction of β-keratin from chicken feathers through the application of various reducing agents, namely sodium metabisulfite (SMB), sodium bisulfite ($NaHSO_3$), sodium sulfide (Na_2S), and thioglycolic acid ($HSCH_2COOH$), with respective extraction yields of 87%, 84%, 53%, and 75% [8]. Other reducing agents, such as 2-mercaptoethanol and dithiothreitol (DTT), have also been reported to produce extraction efficiencies of 83.8% and 77.6%, respectively [9]. A mixture of SMB and urea ($CO(NH_2)_2$) with SDS extracted 28.1%, sodium hydroxide (NaOH) yielded 11.8%, cysteine resulted in 17.4% of β-keratin, and a mixture of SMB, urea, and Na_2S obtained 19.5% [10]. Furthermore, a urea-based mixture showed an extraction efficiency of 50.6% for β-keratin, a combination of Na_2S and NaOH yielded 41.5%, while an SDS and $NaHSO_3$mixture achieved an extraction of 18.3% [11]. These findings highlight the potential of utilizing poultry feather waste as an eco-friendly source of β-keratin through an extraction process involving SMB and SDS as reducing agents to minimize the negative impact of this waste on the environment.

Materials and Methods

Materials

In this study, β-keratin was extracted from chicken and duck feather waste collected from the Silir Chicken Market in Surakarta, Central Java on December 2-5, 2021. Sodium dodecyl sulfate (SDS) and sodium metabisulfite (MBS) used for the reduction process were acquired from Merck. The pH of the solution was maintained in the range of 10-13 using sodium hydroxide (NaOH) obtained from Sigma Aldrich. Universal pH paper was utilized for this purpose. Furthermore, the precipitation procedure employed a commercial ammonium sulfate ($(NH_4)_2SO_4$) purchased from the Muda Berkah Jogja e-commerce store, along with deionized water from the One Lab WaterOne brand.

Instrumentations

Several instruments were utilized in this study, namely: ATR-FTIR spectrometer (Agilent Cary 630), HPLC system (Thermo Fisher), and Freeze Dryer (Telstar LyoQuest).

Extraction of β-keratin from chicken and duck feathers

The collected poultry (chicken and duck) feather wastes were washed with soapy water and dried at room temperature. The dried poultry feathers were weighed and crushed with a blender. About 10-50 mL of SDS at various concentrations of 0.2 M, 0.5 M, and 1 M was added to 0.25-1.25g of poultry feathers, then the solution was heated to a temperature of 30 ºC while the pH was maintained at around 10-13. After that, the solution was stirred for 6 hours and filtered using filter paper until a clean filtrate was obtained, which was then centrifuged at 10000 rpm for 5 minutes to form a supernatant liquid. The whole process was repeated using SMB at the same concentration variations, namely 0.2 M, 0.5 M, and 1 M, as the reducing agent.

The $(NH_4)_2SO_4$ solution was added slowly to the resulting chicken feather filtrate with a 1:1 ratio, then this mixture was centrifuged at 10.000 rpm for 5 minutes to isolate solid particles. This procedure was repeated using duck feather filtrate. Next, deionized water was added to the obtained solid particles and stirred until dissolved. The mixture was then freeze-dried until a solid was obtained. The brown-colored β-keratin in the solid particles was separated using a spatula from the remaining white $(NH_4)_2SO_4$.

Yield analysis

The yield is calculated by dividing the weight of β-keratin by the weight of the feather waste sample and expressed in percent (%) as shown in the following equation 1.

$$yield = \frac{(mass\ of\ \beta\text{-}keratin\ obtained)}{(mass\ of\ feathers\ used)} \text{ x } 100\% \quad (1)$$

Identification of β-keratin structures by ATR-FTIR

The chemical structure of β-keratin was analyzed using Attenuated Total Reflectance Fourier-transform Infrared Spectroscopy (ATR-FTIR) at Sebelas Maret University, in Surakarta, Indonesia. The ATR-FTIR analysis was carried out in transmission mode to collect spectra in the wavenumber range of 4000 cm^{-1} to 650 cm^{-1}. The wavelength of β-keratin obtained in previous study was compared with the FTIR spectra of keratin extracted from feathers using sodium sulfite (Na_2S).

Identification of amino acid composition of β-keratin

The amino acid composition of β-keratin can be determined using High-Performance Liquid Chromatography (HPLC). For this purpose, 4 mL of 6 N HCl was added to 60 mg of β-keratin sample, then the mixture was heated for 24 hours at 110 ºC. Afterwards, the mixture was cooled to room temperature and neutralized (pH 7) with 6 N NaOH. Aquabides was then added to the sample to a volume of 10 mL and filtered with 0.2 mm Whatman filter paper. Next, 50 µL of the sample was added to 300 mL of OPA (Orthophalaldehyde) solution and stirred for 5 minutes, then 10 µL of the mixture was inserted into the HPLC injector L. The HPLC instrument was operated at room temperature, LiChrospher 100 RP-18 (5mm) column, an eluent flow rate of 1.5 mL/min, and mobile phases of A= CH_3OH:50 mM CH_3COONa:THF (2:96:2) pH 6.8 and B = 65% CH_3OH using isocratic elution. Furthermore, detection was performed using a Thermo Ultimate 3000 RS Fluorescence Detector, with an excitation wavelength of 300 nm and an emission wavelength of 500 nm.

Results and Discussion

Extraction of β-keratin from chicken and duck feathers

β-keratin is insoluble in water with low chemical reactivity, thereby requiring high temperature and reducing agents to increase its solubility. In the process of reducing β-keratin in poultry feathers, 2 M NaOH needs to be added so that the pH can be maintained at 10-13. NaOH functions to form an alkaline atmosphere needed in the reduction process that cannot occur in an acidic environment; without an alkaline state, protons cannot be released, thus being unable to break ionic bonds. In an alkaline state, on the other hand, protons are removed from the amino group, and the ionic bonds formed by the electrostatic attraction of the NH_3^+ group of the diamino acid and the COOH group of the dicarboxylic acid can be broken [7].

Severe extraction conditions, such as extremely high/low pH at prolonged exposure and high temperature, produce β-keratin with low molecular weight. Conversely, extraction of β-keratin under mild conditions (i.e., at a temperature of 30 ºC) will break the

disulfide bonds of the protein fibers without significantly breaking the polypeptide bonds, resulting in a stable micromolecular structure of β-keratin with a high molecular weight [8]. To extract β-keratin properly, the reduction process takes 6 hours so that the reducing agent can penetrate deeper into the cell wall. An extraction time of 6 hours has a better effect on the yield of β-keratin compared to an extraction time of 4 hours. The longer the extraction time, the higher the yield of β-keratin [12]. In this study, the reduction reaction of β-keratin is shown in equation 2 and Figure 1 [13].

$$SMB \rightarrow 2Na^{+} + SO_3^{2-} + SO_2 \quad (2)$$

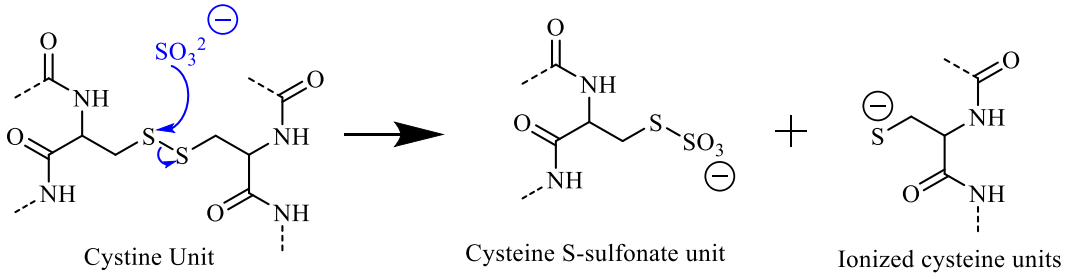


Figure 1. Reduction reaction of β-keratin from poultry feathers using SMB

During the reduction process, β-keratin undergoes heterolytic cleavage of disulfide bonds (-S-S-). The disulfite anion (SO_3^{2-}) is a weak nucleophile, whereas the sulfur atom is a relatively weak reaction center. Nucleophiles must react covalently with electrophilic groups in the protein to become sensitized. The only significant electrophilic groups in proteins are the disulfide bonds of the cystine units that can act as weak electrophiles. The SO_3^{2-} anion attacks one of the sulfur atoms in the S–S bond and breaks it heterolytically, with the thiolate anion of the cysteine unit acting as the leaving group [13].

Reduction process is closely related to a decrease in the oxidation number due to the breaking of disulfide bonds that can affect the structure of the cysteine obtained. In the reduction process of β-keratin, the S atom in the cysteine unit, which has an oxidation number of +4, will experience a decrease in its oxidation number to -1 in the ionized cysteine unit. Disulfide bonds that are disrupted by sulfites will produce cysteine thiols (reduced β-keratin) and cysteine-S-sulfonate residues (Bunte salt), where β-keratin-Cys-S- is reduced keratin and β-keratin-Cys-SSO_3- is Bunte salt [9]. In this study, precipitation of the extracted filtrate was carried out with $(NH_4)_2SO_4$ in high concentration. The addition of salt in high concentrations causes water molecules that were originally bound to the hydrophobic surface of the proteins to bind to the salt. The large number of water molecules that bind to salt ions results in the proteins interacting with each other, aggregating, and precipitating (salting out). In this regard, divalent salts such as $MgCl_2$, $MgSO_4$, and $(NH_4)_2SO_4$ are more effective for protein precipitation than monovalent salts such as NaCl and KCl. $(NH_4)_2SO_4$ is often used in protein precipitation because of its high level of solubility, non-toxicity, and low price [14].

Centrifugation of poultry feathers reduced with SMB produces a brown precipitate at the bottom of the tube, which is β-keratin. Meanwhile, centrifugation of feathers reduced with SDS will form 2 layers; the thick brown liquid in the upper layer is β-keratin, whereas the particle-free supernatant liquid in the lower layer is clear yellow, indicating that β-keratin has been separated from impurities. Additionally, the result of centrifugation of feathers reduced with SDS has a different shape from that of feathers reduced using SMB since the surfactant molecules form a micelle structure on the hydrophobic side of the protein. With the formation of the β-keratin-SDS complex and the prevention of protein aggregation, reduction process using SDS is considered faster and more efficient [9]. SDS prevents the aggregation of polypeptide chains by

blocking the formation of new cross-links, thus increasing the extraction rate and stability of the extracted β-keratin [8]. In this present study, the reduction process was repeated 3 times to increase accuracy; the more reduction processes carried out, the higher the accuracy and validity of the results obtained. The resulting β-keratin solid is brownish white, as presented in Table 1.

Table 1. Extracted β-keratin solids

Chicken Feathers						**Duck Feathers**					
SMB			**SDS**			**SMB**			**SDS**		
0.2 M	0.5 M	1 M	0.2 M	0.5 M	1 M	0.2 M	0.5 M	1 M	0.2 M	0.5 M	1 M
											

Yield analysis

The yield of β-keratin is influenced by several factors, including the concentration of the reducing agent used in the extraction process. SMB concentrations above 0.2 M will decrease the percentage of β-keratin yield as most of the disulfide bonds are broken [16]. There is also a loss of β-keratin during the extraction process, which can be caused by β-keratin not settling completely or remaining in the container when changing or washing. Furthermore, when the pH is too high or too alkaline, β-keratin typically cannot maintain its structure. In addition, hair removal with hot water (high temperature) causes non-reversible denaturation where β-keratin does not regain its original structure [17]. The yields of β-keratin extracted from chicken and duck feathers in this study can be seen in Table 2.

Table 2. Yields of β-keratin extraction at various concentrations of SMB and SDS

SMB Concentration (M)	**Yield (%)**			
	Chicken Feathers		**Duck Feathers**	
	SMB	**SDS**	**SMB**	**SDS**
0.2	76.9	25.4	37.4	39.8
0.5	32.7	19.3	14.3	38.6
1	45.6	15.3	32.5	10.8

In this study, the highest yield was achieved in the extraction of β-keratin from chicken feathers using 0.2 M SMB, which reached 76.9%. This finding is consistent with a previous study which found that chicken feathers reduced using 0.2 M SMB produced a yield of 87.6% [15]. On the other hand, the yield of β-keratin extracted from duck feathers with SDS at a concentration of 0.2 M as the reducing agent had the largest percentage (39%). Therefore, SDS was proven more effective as a reducing agent for extracting β-keratin from duck feathers.

Identification of β-keratin structures by ATR-FTIR

To measure the effect of the extraction process using SMB, the FTIR spectra of β-keratin extracted from chicken and duck feathers using SMB in different concentrations of 0.2 M, 0.5 M, and 1 M were compared, as shown in Figure 2.

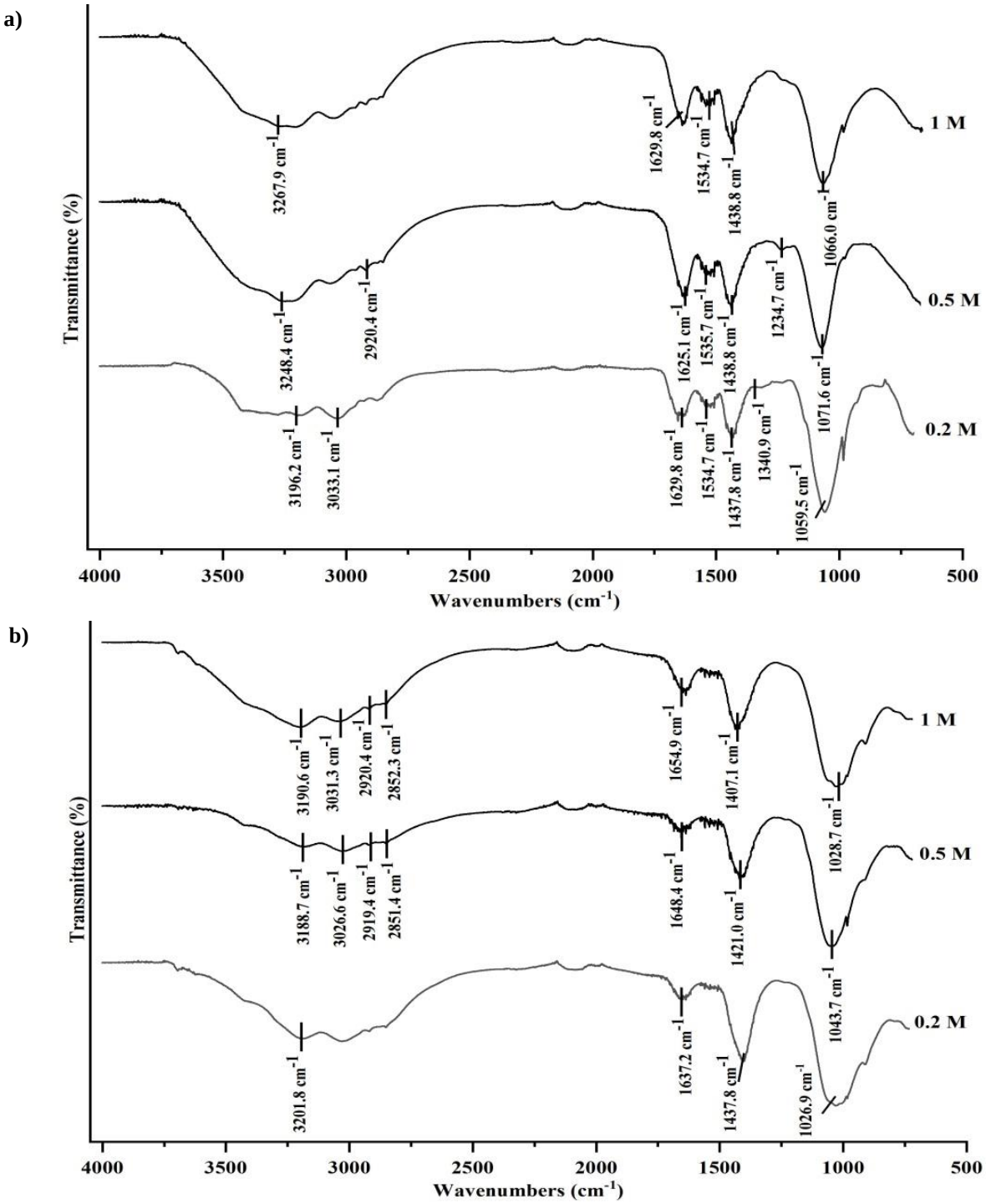


Figure 2. FTIR spectra of β-keratin using SMB reducing agent; a) chicken feathers; b) duck feathers

Similarly, to assess the effects of the extraction process using SDS, the FTIR spectra of β-keratin extracted with SDS in different concentrations of 0.2 M, 0.5 M, and 1 M were compared, as shown in Figure 3.

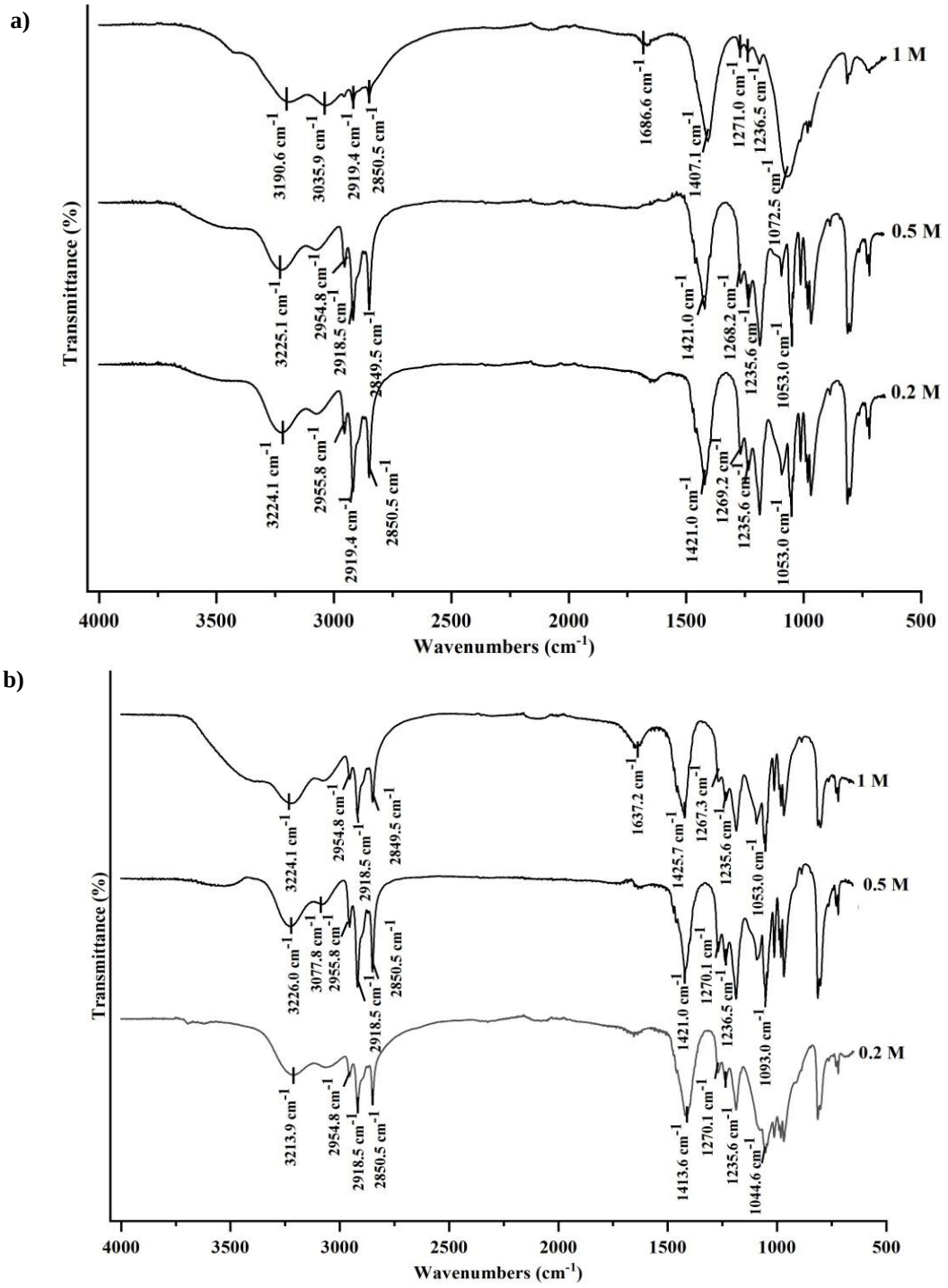
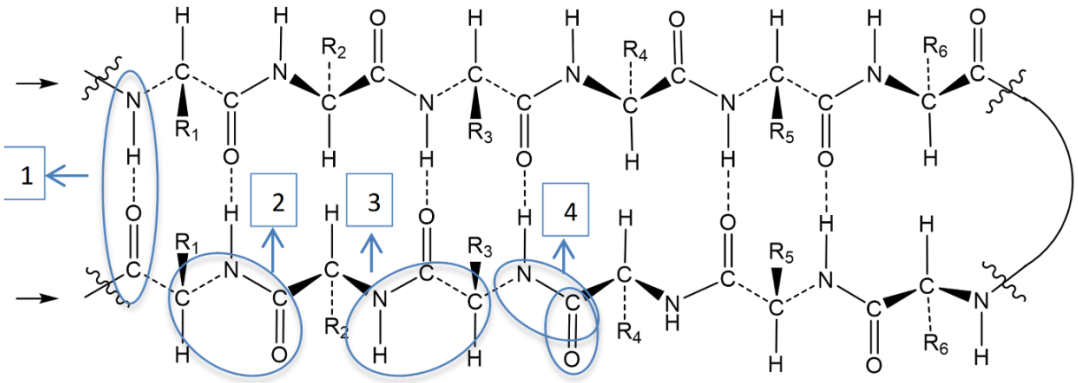


Figure 3. FTIR spectra of β-keratin using SDS reducing agent; a) chicken feathers and b) duck feathers

As seen in Figures 2 and 3, the absorption peaks of β-keratin extracted from chicken and duck feather waste using both SMB and SDS had similar characteristics at the three different concentrations. β-keratin is a protein with unique absorption properties of the peptide bonds (-CONH), and the vibrations of these peptide bonds come from amide A,

amide I, amide II, and amide III bands. The β-sheet structure of β-keratin is displayed in Figure 4.



Notes: Amide A = 1; Amide I = 2; Amide II= 3; Amide III= 4

Figure 4. β-sheet structure of β-keratin

The peaks that appear in the wavenumber range of 3200 to 3500 cm^{-1} indicate amide A and an α-helix structure. N-H and O-H stretching vibrations are insensitive to the backbone of β-keratin, but susceptible to the strength of hydrogen bonds [18]. On the other hand, the absorption bands in the range of 3000 to 2800 cm^{-1} exhibit C-H stretching vibrations and are associated with symmetric CH_3 strains. Such absorption is antisymmetric from the C-H bond in CH_3, where the side chains of amino acid residues in the primary protein chain are saturated, thus being insensitive to structural changes [19].

The extraction process affects the chemical structure of β-keratin. In this regard, the use of SMB at higher concentrations (1 M) to extract β-keratin from chicken and duck feathers causes structural damage to β-keratin as shown by the absence of an absorption pattern at wavenumbers ranging from 2800 to 3000 cm^{-1}. Contrarily, in the extraction process with SDS at high concentrations (1 M), an absorption pattern appears because the reaction using surfactants leads to the formation of by-products in the form of C=O groups [20].

Amide bands I–III provide crucial information about the conformation and structure of the protein backbone. The split peak from 1700-1600 cm^{-1} (Amide I region) corresponds to the C=O stretching vibrations and originates from the combination of α-helix and β-sheet [4]. C=O stretching vibrations are the most sensitive part of proteins in determining secondary structure. These bonds are directly related to the conformation of the backbone, and each type of secondary structure has different C=O strains that can change the conformation of proteins and polypeptides [21].

The amide II region which originates from N-H bending and C-H stretching, signifying absorption bands in the range of 1580 and 1480 cm^{-1}, gives information about the vibration bands of the protein backbone and is related to the β-sheet structure by resulting in lower sensitivity to changes in protein conformation, compared to the amide I band [22]. The absorption band with a weak peak of around 1300 to 1220 cm^{-1} is in the amide III region, which is caused by a combination of C-N stretching and N-H in-plane bending as well as some contributions from C-C stretching and C=O bending [4]. The absorption pattern of amide III as shown in Figure 4 is not visible because most of the disulfide bonds have been broken at SMB concentrations above 0.2 M [16].

The absorption band in the range of 1000 to 1200 cm^{-1} is associated with the asymmetric and symmetric S–O stretching vibrations of the cysteine-S-sulfonate residues formed from the reaction of sulfite and cysteine during the protein extraction process [23]. The sharp

peak also indicates an increase in the content of cysteine-S-sulfonate residues in β-keratin [24]. However, because signals from the disulfide bonds (S–S) cannot be detected by FTIR spectroscopy, the use of Raman spectroscopy is highly necessary to verify these bonds. Absorption of disulfide bonds (S–S) in Raman will appear at 510 cm^{-1} [25]. Based on the FTIR spectra of β-keratin above, β-keratin extracted from both chicken and duck feathers using SDS has a better absorption pattern as SDS itself can prevent the aggregation of polypeptide chains by blocking the formation of new cross-links in the protein, thus increasing the extraction rate and structural stability of the extracted β-keratin [8].

Identification of amino acid composition of β-keratin by HPLC

The process of analyzing the amino acid composition of β-keratin using HPLC was carried out in several stages: sample preparation, standard solution preparation, derivatization, and injection. The preparation of β-keratin began with a hydrolysis process using HCl. The data obtained from this process were in the form of area, retention time, and amino acid levels. The Figure 5 displays the chromatograms of β-keratin extracted from chicken and duck feathers.

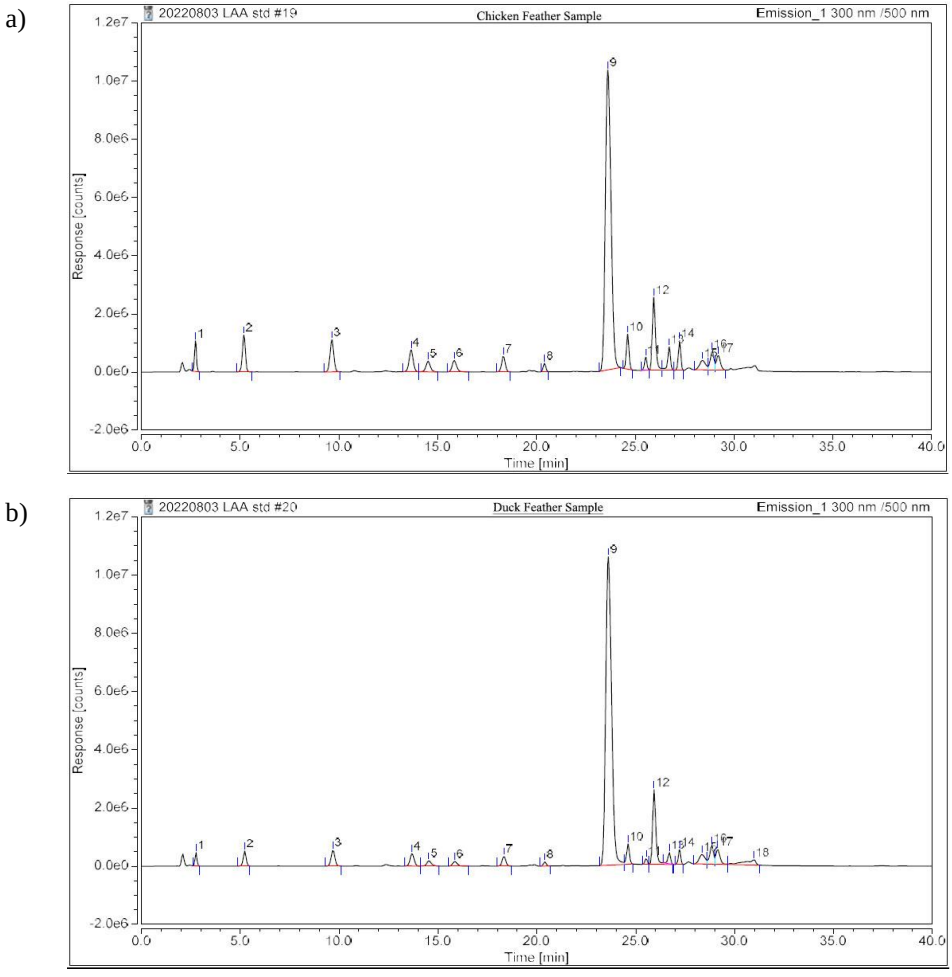


Figure 5. HPLC chromatograms of β-keratin from; a) chicken feathers and b) duck feathers

Based on the chromatograms, the type of amino acids contained in β-keratin in chicken feathers can be determined by comparing the obtained retention time with amino acid standards. Meanwhile, to find out the levels, calculations were carried out using the standard curve functions for amino acids. The amino acids that

make up β-keratin in chicken and duck feathers are: 1) aspartic acid; 2) glutamic acid; 3) serine; 4) glycine; 5) threonine; 6) arginine; 7) alanine; 8) tyrosine; 10) valine; 11) phenylalanine; 13) ileucine; and 14) leucine. The calculation results showed that the highest concentration of amino acids in chicken feathers is glutamic acid at 24.61 ppm. This is in line with a previous study which mentioned that glutamic acid is the most abundant amino acid in chicken feathers [11]. Meanwhile, the highest amino acid content in duck feathers is serine at 11.15 ppm. This finding also supports another prior study which stated that serine is the most abundant amino acid in duck feathers [26]. However, peaks 9, 12, 15, 16, 17, and 18 in Figure 5 do not indicate the types of amino acids that make up β-keratin in chicken and duck feathers but are rather considered as impurities.

Conclusion

The optimal concentration of reducing agent for the extraction of β-keratin was 0.2 M for both chicken feather waste and duck feather waste. The utilization of SMB in the extraction process of β-keratin from chicken feather waste produced the highest yield of 76.9%. Meanwhile, extracting β-keratin from duck feather waste using SDS resulted in a maximum yield of 39.8%. These findings indicate that SMB is preferred as reducing agent for the extraction of β-keratin from chicken feathers, whereas SDS is more effective for duck feathers.

Acknowledgement

This study is supported by the Grant Research Group with contract number 228/UN27.22/PT.01.03/2023

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