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Steam Flash Explosion Assisted Dissolution of Keratin from Feathers

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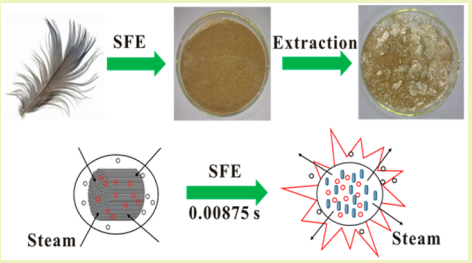
ABSTRACT: A steam flash explosion (SFE) process followed by alkali extraction was applied for extracting keratin from duck feathers. Duck feathers were exploded at different steam pressures (1.4−2.0 MPa) for 0.5−5 min. Results showed that combined SFE with alkaline treatment could effectively promote the dissolution of feather keratin. The optimal extraction conditions in this study were 0.4% NaOH, a solvent to material ratio of 20:1 (v/w), 25 °C, and 1 h. An extraction rate of feathers of 65.78% and a yield of keratin of 42.78% was obtained when the steam exploded feather (1.6 MPa, 1 min) was treated under the above conditions. The extracted keratin retained most of the protein backbone, with the breakage of disulfide cross-links and hydrogen bonds. It was also found that the dissolution involved the fragmentation of macromolecular chains and a loss of some ordered structure. SFE assisted dissolution of keratin from feathers, without the use of harmful reagents, offers a possibility of large-scale exploitation.



KEYWORDS: *Steam flash explosion, Feather, Keratin, Extraction, Urea−SDS−PAGE*

INTRODUCTION

Feathers, containing about 90% keratin, are obtainable in large quantities in the poultry industry. It is estimated that 3−4 billion pounds of feathers are produced annually as a byproduct in the United States[1] and more than 1.5 billion pounds in China.[2] However, they have limited applications on an industrial scale except for animal feed and are mostly disposed by means of incineration or landfilling.[3] Feathers are readily available, low cost, renewable, and biodegradable, making them dependable biopolymer feedstock that should have similar properties to polyamides.[4,5] In recent years, researchers are looking for new applications of feather materials, such as food or compostable packaging,[2,6] edible film,[7] biosorbents,[8] and other composites.[9]

Keratins have plenty of intermolecular and intramolecular cystine cross-links, which distinguishes them from other fibrous proteins such as collagen and myofibrillar protein.[10] Due to the massive disulfide bond cross-linking and the tight packing β-sheet in the polypeptide chain, the dissolution and processing of keratin in common solvents is difficult. Although it can be achieved in a number of ways, including reduction, oxidation, and sulfitolysis of the disulfide bonds, the reagents used in these reactions are often toxic and not friendly to the environment.[11] Recently, several research groups have attempted to dissolve keratins in ionic liquids.[12,13] However, the protein recovery is very little,[14] and much more fundamental research is needed to assess the technology. Hence, investigation into simple and environmentally sustainable methods to extract keratin seems justifiable.

Recently, steam flash explosion (SFE) has received recognition as a green hydrolysis process of protein extraction for the development of biobased materials.[15] The process is based on exposing the biomass to high temperature steam and forcing the steam into the tissues and cells of biomass, followed by explosive decompression completed in milliseconds.[16,17] Our previous study found that disulfide bonds in the feather fibers could be cleaved effectively by SFE treatment only with water.[3] The obtained exploded material mainly showed the presence of shapeless particles due to the strong thermal and mechanical tearing effect. Alkaline solutions have been used extensively to extract protein from agricultural sources, due to the fact that the high alkaline concentration is able to disrupt and dissociate hydrogen from carbolic and sulfate groups in the protein, leading to an enhanced solubility.[18] Even though feather keratin could be extracted easily under severe alkaline conditions (0.25 N NaOH, 120 °C or 1 N NaOH, room temperature),[19,20] it would result in a serious breakage of peptide chains.

The objective of the work was to study the feasibility of extracting keratin from duck feathers using a SFE assisted alkaline method. In this study, the effects of SFE and various operation parameters on the extraction rate and the yield of keratin were investigated, including solvent to material ratio, alkali concentration, extraction time, and temperature. Furthermore, the structural and chemical changes of the extracted keratin were also studied.

EXPERIMETAL SECTION

Materials. The cleaned duck feathers used were provided by Hangzhou Venus Biological Nutrition Co., Ltd., China. The raw feather materials were air-dried to 6% moisture content without any physical crushing processing. All the chemicals were of analytical grade.

SFE Treatment. SFE treatment was performed on a QBS−200B SFE test bed (Gentle Science and Technology Co. Ltd., China). The

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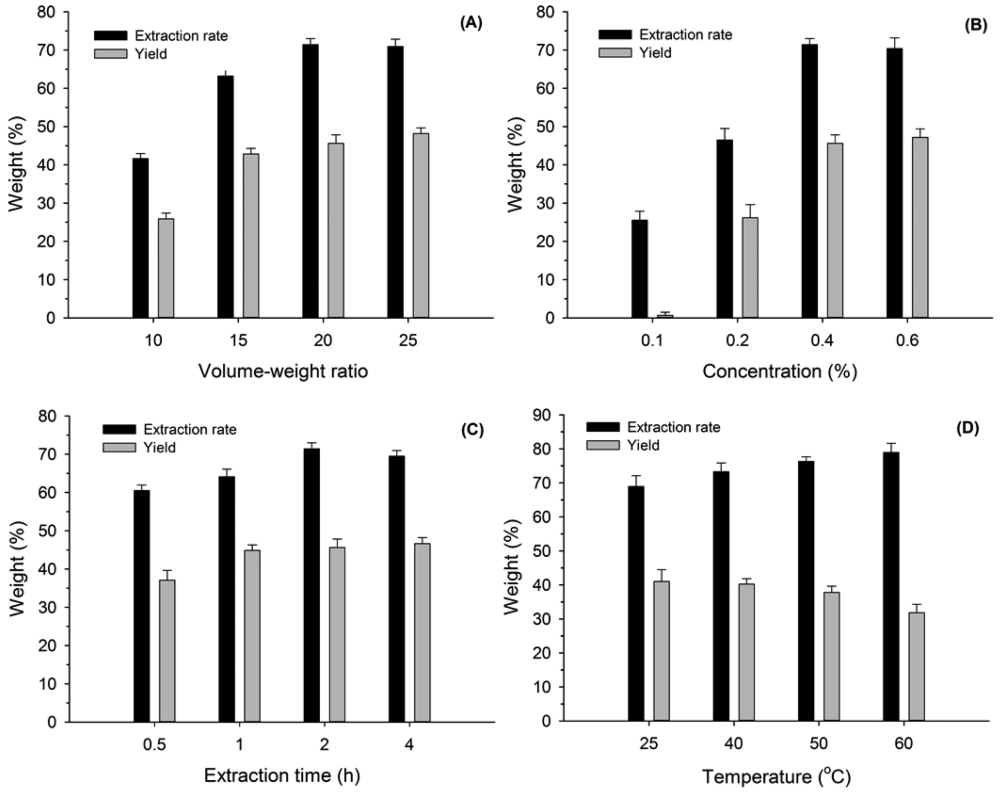

Figure 1. Effect of various factors on the feather keratin extraction using the steam flash explosion assisted alkaline method: (A) effect of volume-weight ratio of solvent and feather, (B) effect of alkali concentration, (C) effect of extraction time, and (D) effect of extraction temperature.

apparatus consists of a steam generator, a receiver, a piston drive system, and a 5 L material chamber with a maximum operational pressure of 4.0 MPa. The whole feathers (~100 g) were put into the chamber, treated at a steam pressure of 1.4–2.0 MPa for 0.5–5 min with saturated steam, and finally terminated by explosive decompression within 0.1 s.[3] All treatments were duplicated. The exploded materials were collected and dried in a vacuum oven at 40 °C for 12 h and then ground with an IKA A11 basic analytical mill (IKA, Germany) to pass through a 0.25 mm diameter mesh for protein extraction.

Keratin Extraction. The ground exploded samples were put into NaOH solution and stirred continuously at 25–60 °C for 0.5–4 h under magnetic stirring conditions according to the method of Li and Lee[21] with some modifications. The insoluble feather residues were removed by centrifugation at 4000 rpm for 10 min. Keratins dissolved in the supernatants were obtained by adjusting the pH value to 4.5 using 2 N HCl at room temperature. The residues and the recovered keratin were both washed three times with distilled water, dried at 50 °C, and weighted. The extraction rate of feathers was expressed as the difference between the initial weight of the sample and the residue divided by the initial weight of the sample. The keratin yield was expressed as the weight of keratin divided by the initial weight of the sample. The obtained keratin materials were pulverized and kept at 4 °C before further analysis.

Size Exclusion HPLC. The alkaline extract of the feather material exploded at 1.6 MPa was applied to a HITACHI–L2000 HPLC system (Hitachi, Japan) fitted with a TSK–gel G3000 SW_{XL} column (0.75 cm × 30 cm) with a TSK–gel G2000 SW_{XL} guard column (0.75 cm × 7.5 cm). The extract was diluted 10 times and equilibrated for 3 h using three different buffers (buffer a, 0.05 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.2; buffer b, buffer a plus 6 M urea; buffer c, buffer a containing 6 M urea and 5 mM β-mercaptoethanol (β-ME)).[22] Then the sample was injected (20 μL) onto the column after filtering with a 0.22 μm pore size membrane filter. Elution was carried out at 0.6 mL/min and detected at 280 nm.

Urea–SDS–PAGE. The raw feather and the extracted keratin from different steam pressures were dissolved in 0.05 M Tris–HCl (pH 8.0) containing 8 M urea and 5% β-ME to reach a 4 mg/mL final concentration and then mixed with 2× sample buffer (20% glycerol, 4% SDS, 5% β-ME, 0.5 M Tris–HCl, pH 6.8). Then, 8 μL of the sample was loaded into each well. Urea–SDS–PAGE was carried out with a Mini-Protein Tetra system (Bio-Rad Lab. Inc., USA) with a 5% stacking gel and 15% separating gel containing 6 M urea. The separation was performed at 80 V and followed at 120 V and then stained with Coomassie Brilliant Blue (R-250), followed by destaining with 10% acetic acid and 10% methanol until a clear background was observed.

Fourier Transform Infrared (FT–IR) Spectroscopy. Infrared spectra of the raw feather and the extracted keratin, oven-dried at 105 °C for 2 h, were measured by a Thermo Nicolet iS10 spectrometer coupled with a zinc selenide attenuated total reflection (ATR) crystal. The spectrum of each sample was acquired in the 4000–600 cm^{-1} region at 4 cm^{-1} resolution with 50 scans by averaging the repeated measurement data. Spectra were baseline-corrected and normalized to the amide I band by using the OMNIC software package (Thermal Scientific, version 8.2). The regions of amide III were curve-fitted with Gaussian bands using PeakFit 4.12 software (SeaSolve Software Inc.).

Differential Scanning Calorimetry (DSC). DSC measurements were made under a nitrogen flow (50 mL/min) with a DSC Q200 series (TA Instruments) calibrated with indium. The samples (~2 mg) were loaded into aluminum pans and then heated at a rate of 10 °C/min from 40 to 290 °C. All experiments were performed in duplicate.

Thermogravimetric Analysis (TGA). Thermogravimetric measurements were performed on a Mettler–Toledo TGA/SDTA851e under a dynamic atmosphere of nitrogen at 20 mL/min between 25 and 600 °C at 10 °C/min. The samples were first dried in a vacuum oven at 60 °C. Samples (~5 mg) were placed in each aluminum crucible, and the data were analyzed using Mettler–Toledo STARe System.

Statistical Analysis. Results were presented as the mean ± standard deviation. One-way analysis of variation (ANOVA) and

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Duncan's multiple range tests at a 5% significance level were used for the data analysis by SPSS software (version 19.0).

RESULTS AND DISCUSSION

Extraction of Keratin from Feathers Assisted by SFE. *Effect of Operation Parameters on Keratin Extraction.* Alkali solutions have been used extensively to extract protein from agricultural sources.[18,23] In this study, NaOH was used as the only extractant, which was able to break down hydrogen bonds and introduce electrostatic repulsion via charged residues, thus leading to an enhanced solubility.[18] The extraction efficiency depends largely on the conditions of extraction, including solvent to material ratio, alkali concentration, extraction time, and temperature. Thus, the effect of the above-mentioned operation parameters on the extraction rate and the yield of keratin from the steam exploded materials (1.8 MPa, 1 min) was investigated.

To determine the effect of the solvent to material ratio, the condition of 0.4% NaOH, 2 h, and 25 °C was selected. As shown in Figure 1A, the extraction rate and the yield of keratin increased with the ratio to reach a maximum at 20:1 (v/w), while it changed not significantly at larger ratios ($P > 0.05$). To study the alkali concentration effect, the experiments were conducted under the conditions of a solvent to material ratio of 20:1 (v/w), 2 h, and 25 °C (Figure 1B). Extraction rates increased significantly when NaOH concentration was raised from 0.1% to 0.4%. It is reported that the protein structure could be more readily opened to expose hydrophobic residues, due to disruption of hydrogen bonds and dissociation of hydrogen from carbonyl and sulfate groups at higher alkaline concentration.[24] Even though feather keratin could be extracted easily under severe conditions (0.25 N NaOH, 120 °C or 1 N NaOH, room temperature),[19,20] it would result in the serious breakage of peptide chains, which was a disadvantage for its application.

The effect of extraction time on the keratin extraction is shown in Figure 1C. In general, a longer extraction time gave a higher extraction rate.[25] However, in the study, the effect of extraction time became evident at the first hours of extraction, after which the yield of keratin changed not significantly. The effect of extraction temperature was conducted at a constant condition of 0.4% NaOH, 2 h, and a solvent to material ratio of 20:1 (Figure 1D). Although a higher temperature treatment was found to be a benefit for the enhancement of keratin extraction, the obvious temperature effect appeared between 25 and 40 °C in the study. When the temperature exceeded 40 °C, the yield of keratin decreased significantly, indicating the keratin would be decomposed at high temperature.[13] After optimization based on extraction rate and yield of keratin, conditions of 0.4% NaOH, 1 h, a solvent to material ratio of 20:1, and 25 °C were used for the extraction process.

Effect of SFE on Keratin Extraction. Figure 2A showed the effect of steam pressure on the protein extraction. After SFE treatment, the extraction rate of feathers increased to 45.20%, 65.78%, 71.42%, and 70.12% with the yield of keratin of 27.46%, 42.78%, 45.13%, and 43.57% at 1.4, 1.6, 1.8, and 2.0 MPa, respectively. No significant differences in the yield of keratin were observed between 1.6 and 2.0 MPa ($P > 0.05$). However, the raw feather materials are hardly dissolved under the above alkaline conditions. After SFE treatment, the increase may be attributed to the increasing electrostatic repulsive force between surface groups of the exploded material that exists mainly in the anion form.[3] The effect of treatment time on

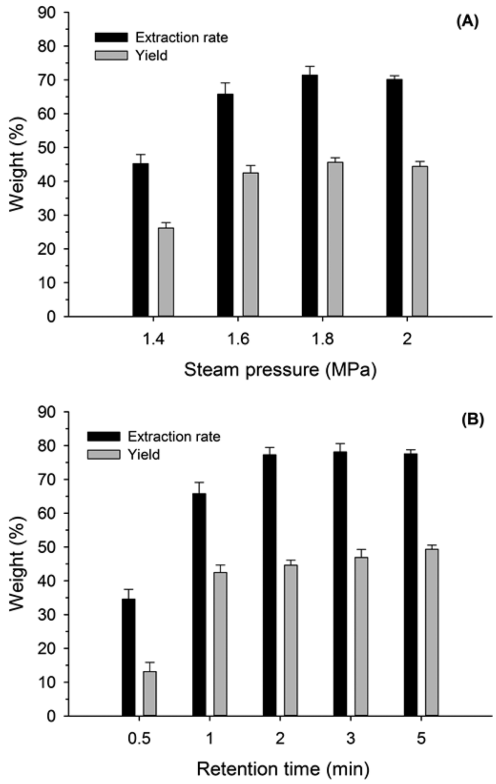

Figure 2. Extraction rate of feather and the yield of keratin at various SFE treatment conditions: (A) treated at 1.4, 1.6, 1.8, and 2.0 MPa for 1 min; (B) treated at 1.6 MPa for 0.5, 1, 2, 3, and 5 min.

protein extraction at 1.6 MPa was shown in Figure 2B. It was found that the extraction rate and yield of keratin reached a maximum at 1 min, after which it changed not significantly ($P > 0.05$). Among all the tested treating time, 1 min was considered to be enough for the SFE treatment.

Due to the extensive disulfide cross-linking and plenty of hydrophobic residues, keratin is extremely difficult to extract.[10] The conventional method for solubilizing keratin is roughly characterized by pulverizing keratin materials to micron-sized filaments, and then hydrolyzing the materials using protein denaturing or reducing reagents (e.g., urea, ionic liquid or DTT).[10,12,26,27] However, these are unsustainable approaches where the used reagents are often toxic and not friendly to the environment. By optimizing the extraction conditions of feather keratin, the researchers obtained a maximum solubility of 55%, 71% and 75%, respectively.[10,26,28] Obviously, the extraction rate of feather (65%−71%) in our study was consistent with the above results obtained by the conventional method. SFE is a physicochemical process with a short processing cycle.[3] In this study, duck feathers were treated by SFE to obtain shapeless particles, and then hydrolyzed at mild alkaline conditions without the use of harmful reagents. Although this method gave a slightly low protein yield (42%), its simple operating procedure, mild extraction conditions, and low environmental impact would make it susceptible to large-scale exploitation.

Characterization of Extracted Keratin. *Size Exclusion Chromatography.* Figure 3 showed the molecular weight distribution of the extracted keratin diluted and eluted in three different buffers. The elution profile could be divided into two regions of high molecular weight (HMW) and low molecular weight (LMW) at 21.2 min, corresponding approximately to 10 kDa according to the calibration with the protein standards. It

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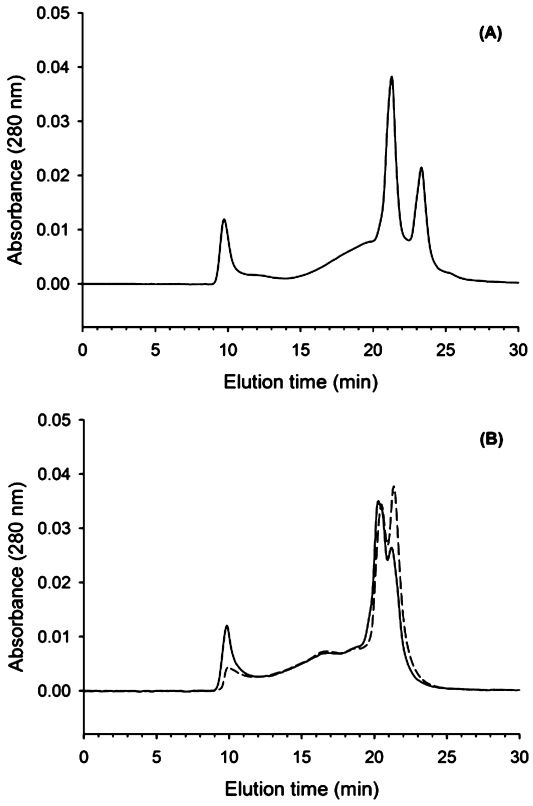

Figure 3. Size exclusion chromatography (TSK−gel G3000 SW_{XL}) of keratin extracted from feather treated by SFE at 1.6 MPa for 1 min. The extracts were diluted and eluted in three different buffers: (A) 50 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.2 (buffer a); (B) buffer a + 6 M urea, pH 7.2 (solid line); buffer a + 6 M urea +5 mM β-ME, pH 7.2 (dash line).

appeared that these abundant HMW particles were representative of protein polymers, indicating that the dissolution of keratin stemmed not merely from the small molecule polypeptides but also from the degraded protein. To illustrate the effect of disulfide cross-links and noncovalent interactions on the keratin extraction, the extract was diluted and eluted in a urea buffer with or without β-ME (Figure 3B). Compared with the SE−HPLC profile obtained with sodium phosphate buffer, elution in urea partially dissolved some HMW material. After incubation with urea plus β-ME, the amount of HMW protein decreased evidently, indicating that parts of the disulfide bonds were retained in the extracted keratin. By using TGA coupled with mass spectroscopy (TGA-MS), some authors also showed that the remaining disulfide bonds in the feather fibers after heat treatment were involved in the accumulation of HMW particles.[29] Obviously, these materials still have great potential to self-assemble into polymers through noncovalent interactions (e.g., hydrogen bonds) and disulfide bonds, which should be useful for a wide range of industrial applications.

As shown in Figure 3B, the cleavage of disulfide bonds in proteins by β-ME resulted in the accumulation of LMW molecules. However, a minor peak is still preserved in the urea plus β-ME extract, indicating the presence of nondisulfide cross-links in the extracted keratin. This may be due to the fact that the resulting particles are very compact, and that the intermolecular disulfide bonds between the monomers are inaccessible to β-ME. Another possible reason is that partial breakage of disulfide bonds of feather fibers during SFE treatment may enhance the interactions between the exposed hydrophobic regions and then form new cross-links that could provide solvent resistance for the protein.[30]

Urea−SDS−PAGE. The molecular weight distribution of soluble proteins in both the raw feather material and treatments was also characterized by Urea−SDS−PAGE (Figure 4). The

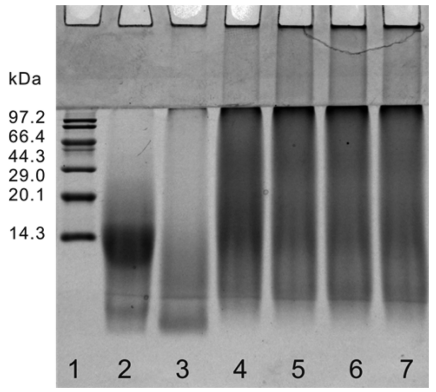

Figure 4. Urea−SDS−PAGE analysis of keratin extracted using the alkaline method. Lane 1: standard protein markers. Lane 2: raw feather. Lane 3: keratin extracted by 1 N NaOH. Lanes 4−7: keratin extracted from feather treated by SFE at 1.4, 1.6, 1.8, and 2.0 MPa for 1 min.

raw feather showed one clear protein fraction at about ∼10 kDa, correlating to feather keratin monomer.[31] The keratin extracted by 1 N NaOH (lane 3) showed a diffuse band of fragmented peptides, which was consistent with the previous studies.[20,32] In this study, feather keratins extracted by alkali assisted by SFE were partially fragmented as evidenced by the appearance of new continuous bands below 10 kDa, resulting in a mixture composed of peptide fragments with heterogeneous molecular masses. On the contrary, when feather materials were treated by superheated water at 220 °C (about 2.3 MPa) for 2 h, the proteins were broken down into discrete molar masses of oligopeptides around 1.8 kDa.[33] The difference must be attributed to the shorter processing time in high temperatures during SFE treatment.

As shown in Figure 4, the extracted keratin showed a continuous background in lanes 4−7, which meant a continuous distribution of keratin related fragments. This may be due to the random cleavage of polypeptides in the keratin during SFE treatment. Meanwhile, the HMW molecules in the extracts were unable to penetrate the separation gel, indicating extra cross-linking occurred through nondisulfide covalent bonds. High temperature and humidity, and tearing effects generated in the explosion phase, involving destabilization of the native structure of feather keratins, could facilitate interactions between proteins to form aggregations. The similar phenomenon was also found in extruded proteins, such as wheat flour protein[34] and fibrous meat.[35]

ATR−FTIR. FTIR investigation can be used to assess the conformational changes in the polypeptide chains caused by thermal−mechanical−chemical processes.[3] The ATR−FTIR spectra of the samples were shown in Figure 5. The raw feather and the extracted keratin showed typical amide vibrations at 3273 cm^{-1} (amide A, N−H stretching), 1600−1700 cm^{-1} (amide I, C═O stretching), 1480−1580 cm^{-1} (amide II, C−N stretching and N−H bending), and 1220−1300 cm^{-1} (amide III, C−O and C−N stretching, O═C−N and N−H

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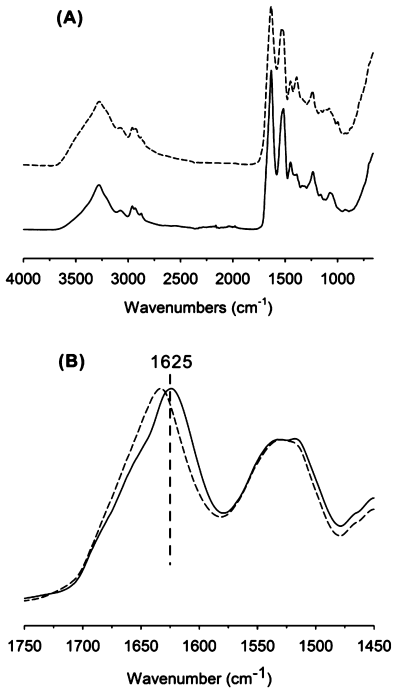

Figure 5. ATR−FTIR spectra (A) and the amide I region (B) for raw material (solid line) and keratin material extracted from feathers treated by SFE at 1.6 MPa for 1 min (dash line).

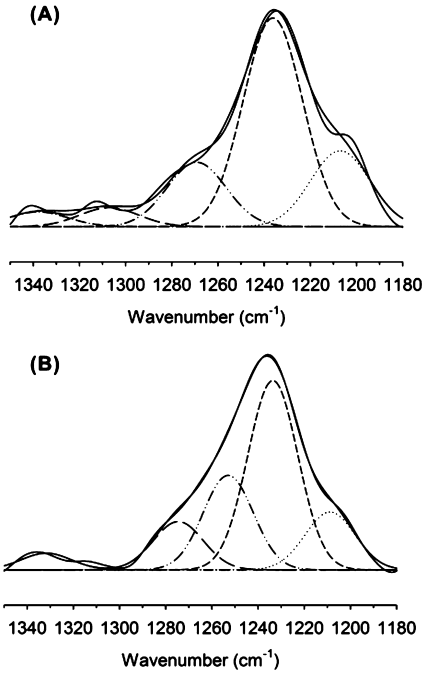

Figure 6. FTIR peak resolution of amide III spectral regions, 1350−1200 cm^{-1}: (A) raw feather; (B) extracted keratin.

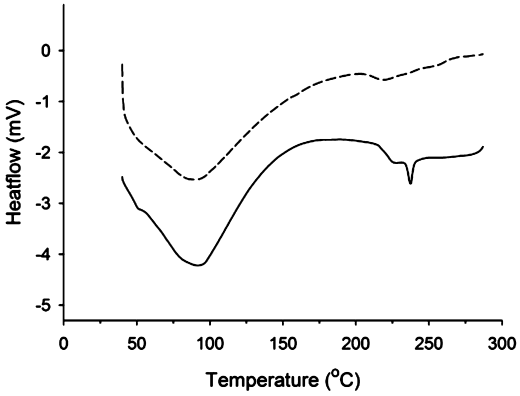

Figure 7. DSC curves of raw feather (solid line) and extracted keratin (dash line).

bending).[12] Figure 5A showed that no new functional groups appeared in the extracted keratin. Therefore, dissolution in the alkali solution did not strongly affect the peptide bonds of the feather keratin. The chemical modifications were further conformed by the changes in the amide I region of the spectrum (Figure 5B). A shift in the peak at 1625 cm^{-1} toward higher wavenumbers was observed in the extracted keratin, indicating the disruption of some ordered β-sheet structures. The results were in general agreement with the findings among the materials treated by ionic liquids. By use of solid state ^{13}C NMR, these authors showed that the chemical changes mainly resulted from the breakage of disulfide cross-links and hydrogen bonds.[11,12]

The secondary structure of the protein could be characterized by the amide I region of FTIR. However, due to atmospheric water vapor interference, the deconvolution of the amide III regions is recommended for more accurate analysis of the protein secondary structure.[8,36] The relative proportions of the microstructural components could be obtained according to α-helix (1330−1295 cm^{-1}), β-turn (1295−1270 cm^{-1}), random coil (1270−1250 cm^{-1}), and β-sheet (1250−1220 cm^{-1}).[8] From Figure 6, the raw feather contained 71.51% β-sheet, 22.02% β-turn, and 6.47% α-helix. The extracted keratin (1.6 MPa, 1 min, 0.4% NaOH) contained 60.99% β-sheet, 14.53% β-turn, and 24.46% disordered regions. The peak analysis revealed that some ordered secondary structures were inclined to convert into unfolded or disordered structures after SFE assisted dissolution and regeneration. These abundant β-sheet structures characterized the keratin materials as feasible starting materials suitable for the development of new biomaterials.

Thermal Stability and Phase Behavior. The phase behaviors of the raw feather and the extracted keratin were studied by DSC (Figure 7). The large endothermic peak at around 100 °C was due to the release of bound water. The raw feather showed a bimodal profile between 225 and 240 °C, mainly resulting from the melting of the β-form crystallites overlapped by the degradation of the cysteine-rich matrix.[37] After hydrolysis, the extracted keratin showed the endothermic peak appeared at lower temperature accompanied by decreased underlying area, indicating the crystallites were partially preserved. The shift mainly arises from the rupture of disulfide and hydrogen bonds that occurs in the extracted keratin. It also resulted in the disruption of ordered structures of keratins, as shown in Figure 6.

The thermal degradation behavior of the raw feather and the extracted keratin was investigated by TGA (Figure 8). The curves showed that the thermal stability of the extracted keratin was well maintained under the experimental condition. Two stages of decomposition could be seen in these materials. The first weight loss step around 100 °C was attributed to the loss of bound water. The second loss in the 250−400 °C range was mainly the result of the degradation of the feather keratin, which was associated with the breakage of the β-sheet conformation and disulfide bond.[4,38]

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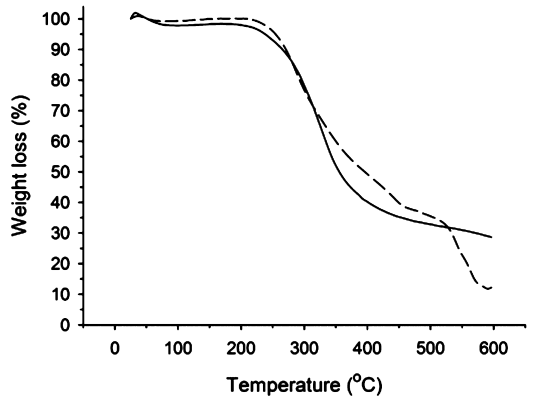

Figure 8. TG curves of raw feather (solid line) and extracted keratin (dash line).

CONCLUSIONS

This study showed that steam flash explosion assisted alkaline method was practical to extract keratin from feathers. An extraction rate of 65.78% was obtained under the optimum extraction conditions. The extracted feather keratin retained most of the protein backbone, with the breakage of disulfide cross-links and hydrogen bonds. It was also found that the dissolution involved the fragmentation of macromolecular chains and loss of some ordered structure. Although the method gave slightly low keratin yield, its simple operating procedure, mild extraction conditions, and low environmental impact would make it susceptible to large-scale exploitation. Further experimental studies with regard to the preparation and characterization of various films from these materials are currently underway.

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Author Contributions

The final version of the manuscript has been approved by all authors.

Notes

The authors declare no competing financial interest.

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