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PAPER

Extracting keratin from wool by using L-cysteine

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Keratin proteins are the major component of hair, feathers, wool and horns and represent an important source of renewable raw materials for many applications. The dissolution of the wool keratin is the first step of reuse of keratin wastes. In this work, L-cysteine was applied to the dissolution of wool keratin for the first time as reducing agent. The dissolution time was 5h at 75°C, with the 72% dissolubility. XRD, ATR-FTIR and ^{13}C NMR showed that the content of α-helix structural in regenerated wool keratin were decreased compared with natural wool. The content of S-S crosslinkages for regenerated wool keratin significantly decreased and broke about 62% of the S-S crosslinkages in the natural wool, as observed from Raman spectra.

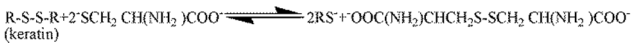
1. Introduction

In recent years, several proteins have been investigated in the development of naturally-derived biomaterials such as silk fibroin, collagen and gelatine. However, few have been reported on keratin, being the major component of hair, feathers, wool, nails and horns of mammals, reptiles and birds, even though it is one of the most abundant proteins. Moreover, keratin wastes are an important source of renewable raw material, since the disposal of organic wastes deriving from farm breeding, wool fibre by-products from textile processing, poor-quality raw wools not fit for spinning, horns, hairs and feathers from butchery have been estimated worldwide more than 5 million tons per year.[1,2] Keratin regenerated from wool has many properties such as biodegradability (degrades both in vitro and in vivo), biocompatibility and self-extinguishing. So it is important to transform the waste wool into new materials to increase its value addition.

Wool is a fibrous protein that contains up to 95% wt of pure keratin.[2] However, wool keratin is tightly packed in α-helices and β-sheets into a super coiled polypeptide chain with a high degree of disulfide cross-linkages, salt bonds, hydrogen bonds and other bonds. Extraction of keratin from wool requires chemical modification of cysteine because unreduced keratin solutions are stable. It has been reported that the regenerated wool keratin can be divided into two parts: the low-sulphur content keratin (60% wt) at molecular weights ranging from 60 to 45 kDa, the high-sulphur content keratin (26% wt) at molecular weights ranging from 28 to 11 kDa. [3]

Various attempts have been made to extract keratin from hair, feather and wool. The chemical methods mostly employ strong acid and alkali hydrolysis and chemical, such as peroxides,[4] sulfites,[5] dithiothreitol,[1] and thiols,[6] cleavage of the disulfide bonds to extract keratins. Although these chemicals methods have many advantages, there are some deficiencies. For example, 2-mercaptoethanol and mercaptoacetic acid are harmful and difficult to handle, the molecular weight of keratin extracted with peroxides or suifites is not big enough. The physicochemical methods, such as high density steam flash-explosion,[7] are an innovative method for pre-treatment of biomass. However, these treatments always need the high pressure apparatus. Other physicochemical methods, for example, superheated water treatments result in severe degradation of keratin. Enzymatic hydrolysis[8] and ionic liquids[9,10] are recently receiving considerable attention. The long production cycle of the enzymes methods has so far limited the development of industrial processes. Moreover, most keratins are converted into much shorter oligopeptides and amino acids. The high prize and the limited reuse time of ionic liquid restrict its application. Thus, researchers have focused on finding simple and eco-friendly processing methods to dissolve and extract wool keratins.

L-cysteine is an α-amino acid with the chemical formula $HOOCCH(NH_2)CH_2SH$. Due to the ability of thiols to undergo redox reactions, we used L-cysteine as the substitute of 2-mercaptoethanol which is a benchmark for extracting keratin because of good yield and undamaged keratin, but environmental harmfulness. Similar to the mercaptoacetic acid,[11] the reaction mechanism is either oxidised to its disulfide form, as shown in Scheme 1, or can in part form a new disulfide bridge $R\text{-}S\text{-}S\text{-}SCH_2CH(NH_2)COO^-$. In either case the keratin disulfide link is cleaved.



Scheme 1

In this article, the dissolution of wool keratin fibres in L-cysteine was studied, and the structures and properties of the regenerated keratin were characterized by SDS gel electrophoresis, X-ray diffraction (XRD), Fourier Transform

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infrared spectroscopy with Attenuated Total Reflection technology (ATR-FTIR), Raman spectroscopy, solid state Nuclear Magnetic Resonance (NMR), and thermogravimetry analysis (TGA).

2. Experimental

Materials

The wool keratin fibres samples were provided by Yuan Heng villi products Co., LTD (Zhejiang Province, P. R. China) and the acetone (C_3H_6O, AR), ethanol (C_2H_6O, AR), L-cysteine ($C_3H_7NO_2S$, BR), urea (CH_4N_2O, AR), sodium hydroxide (NaOH, AR) and acetic acid ($C_2H_4O_2$, BR) were obtained from Sinopharm Chemical Reagent (Shanghai, P. R. China). Dialysis tubing (8 000-14 000 Da) was provided by Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, P. R. China).

Pretreatment of Wool Fibre

The wool keratin fibres used in these experiments were first cleaned by an acetone/ethanol mixture solvent system in a Soxhlet extraction set for 48 h, then rinsed with distilled water, and dried at 100°C for 12 h in an oven prior to solubility experiments.

Fabrication of Keratin Powder

Five grams of cleaned and conditioned fibres were cut into snippets some millimetres long. Put the fibres in 100 ml of aqueous solution containing urea (8 M) and L-cysteine (0.165 M), adjusted to pH 10.5 with NaOH 5M and treated by shaking for 5 h at 75°C.

First the mixture was filtered, dialyzed against distilled water using a dialysis tube (molecular weight cut off 8 000-14 000 Da) for 3 days at room temperature, changing the distilled water four times a day. Then adjusted the pH of the solution to 4.0–4.5, large amounts of solid keratin were precipitated. Finally the mixture was centrifuged and dried in the freeze dryer to obtain recovered pure keratin powder. Thus, obtained keratin was sealed and stored at 4°C before use.

Measurements

Protein samples were diluted in loading buffer (Shanghai Yuanye Bio-Technology) and electrophoresed using the Bio-rad mini vertical electrophoresis system (America Bio-rad) in 4–12% Bis-Tris gradient gels (Shanghai Yuanye Bio-Technology).

The molecular weight of extracted keratin was determined by gel permeation chromatography. It was carried out on a Waters 1525 HPLC (TSKgel 2000 SW_{XL}, 7.8 × 300 mm column). Acetonitrile/Water/Trifluoroacetic acid (V/V: 10/90/0.1) was used as the eluent and the flow rate was 0.5 ml/min. The column temperature was 30°C. A keratin sample of 100 mg was placed in a graduated flask of 10 cm^3 volume, 7 cm^3 of the solvent was added, and then set for 1 hours for dissolution. Next, the keratin solution was mixed by shaking for about 10 minutes, and added the solvent to the volume of 10 cm^{-3}. After mixing and filtration, clear keratin solution were obtained, suitable for the GPC analysis.[12]

FTIR spectra with attenuated total reflectance technique (ATR) were obtained using a Nicolet 6700 FTIR spectrometer equipped with SMART iTR diamond accessory in a spectral scanning frequency range of 650–4000 cm^{-1} at the spectral resolution of 4cm^{-1}.

The samples were cut into small pieces of 1–2 mm in length for the measurements. XRD was performed on a Rigaku-D/Max-2550 PC diffractometer with Cu KR radiation (k=1.54056 Å) at 40 kV, 200 mA in the range of 5–60° at the rate of 0.02°/min.

Raman spectra were recorded on a Raman microscope system (inVia Reflex, England), the laser excitation was provided by an argon ion laser operating at 50 mW of 785 nm output. The laser beam on the sample was focused to a spot diameter of 1μm using a 100× microscope objective. Spectra were recorded by scanning the 200–2000 cm^{-1} region with a total acquisition time of 1000s.

^{13}C NMR spectra were recorded at 400 MHz on a Bruker Avanceu-400 spectrometer. The ^{13}C CP MAS NMR spectra of these samples were acquired using a 4 mm rotor with Kel-f cap at 10 kHz spinning rate. The contact time in the CP MAS experiments was 2.4 ms with a recycle delay of 1 s and cw decoupling. The number of scans was ~90 000 to 100 000.

Thermogravimetry analysis (TGA) measurement was performed on a NETZSCH-TG 209 F1 thermogravimetric analyser. The measurement was performed using 1–5 mg of the sample under an atmosphere of nitrogen gas. The samples were heated from 30 to 900°C at a rate of 10°C /min.

3. Results and discussion

Dissolution of wool fibre

From Fig. 1, we can see that the dissolubility of wool with sulfites,[5] thiols,[6] enzymatic,[8] ionic liquids[10] and L-cysteine. The dissolubility of wool in L-cysteine exhibited better effect than other methods.

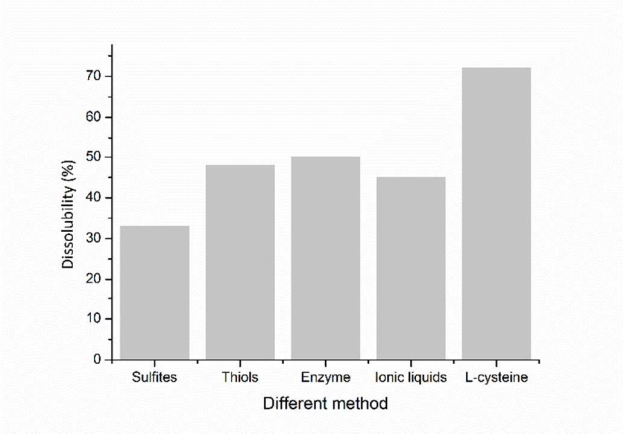


Fig. 1 Dissolubility of wool with different solution.

SDS-PAGE gel electrophoresis was used to estimate the sizes of the isolated protein homologs in Fig. 2. As can be observed, the most intense bands appeared at 10 kDa, 15 kDa, 40 kDa and 60 kDa in the natural wool, whereas in regenerated wool only two bands appeared, at about 40 kDa and 55 kDa. This suggests that the molecular weight does not change during the process of regeneration.

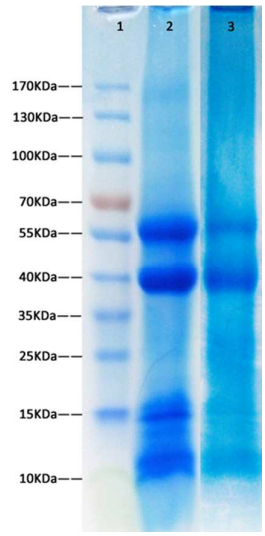


Fig. 2 SDS-PAGE pattern of (1) protein standard (Thermo Scientific, MW 10,000–170,000), (2) natural wool and (3) regenerated wool keratin.

The molecular weight of regenerated wool keratin solution was also examined by GPC. The weight-averaged and number-averaged molecular weights of regenerated keratin were 18,905 and 16,877 Da respectively. The polydispersion degree was at the level of 1.120. The difference between the results by GPC and SDS-PAGE gel electrophoresis maybe lies in the high molecular weight part of regenerated keratin is insoluble in the solvent which fulfil the demands for eluents in the GPC method.

Characterization of regenerated wool keratin

XRD studies. The X-ray diffraction (XRD) spectra of the natural wool and regenerated wool materials are presented in Fig. 3. Two crystal structures are typically observed for natural wool and regenerated wool. The α-helix structure is shown in peaks at 2θ=9° and 17.8° whereas the β-sheet structure manifests peaks at 2θ=9° and 19°.[9,10,13] Compared to the natural wool, the XRD patterns for the regenerated wool clearly show the decreasing of the peak at about 9° and the enlarging of the peak at about 19.50°. This is because that the α-helix structure is destroyed by the L-cysteine during the dissolving process and is not restored.[10] The peaks at about 9° and 19° are both significantly stronger in the regenerated wool, suggesting a slightly increase of the content of the β-sheet structure.

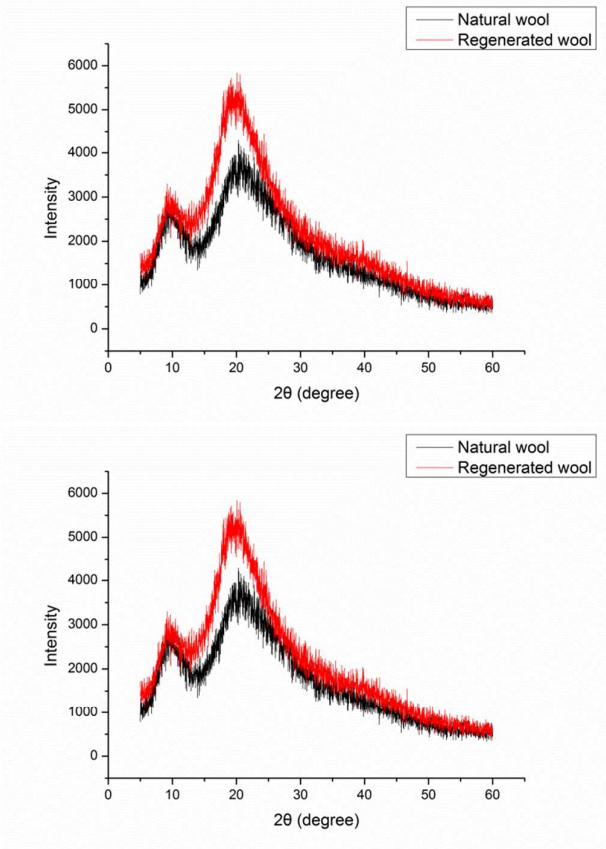


Fig. 3 XRD of natural wool and regenerated wool.

ATR-FTIR studies. The ATR-FTIR spectra of natural wool and regenerated wool are shown in Fig. 4. The spectra show characteristic absorption bands ascribed to the peptide bonds (–CONH) and these have been labelled as Amide A, Amide I, Amide II and Amide III bands. An absorption peak at 3295 cm^{-1} is assigned to N–H stretching (Amide A). A strong absorption peak at 1651 cm^{-1} should be ascribed to C=O (Amide I). A medium strong peak is observed at 1519 cm^{-1} and assigned to C–N stretching and N–H in-plane bending vibrations (Amide II), while the weak band in the range of 1238–1240 cm^{-1} is related to the C–N and C–O stretching vibrations (Amide III).[10,11,14,15] No additional bands are seen in the ATR-FTIR of the regenerated samples, which means that the regenerated wool keratin by L-cysteine does not affect the peptide bonds.

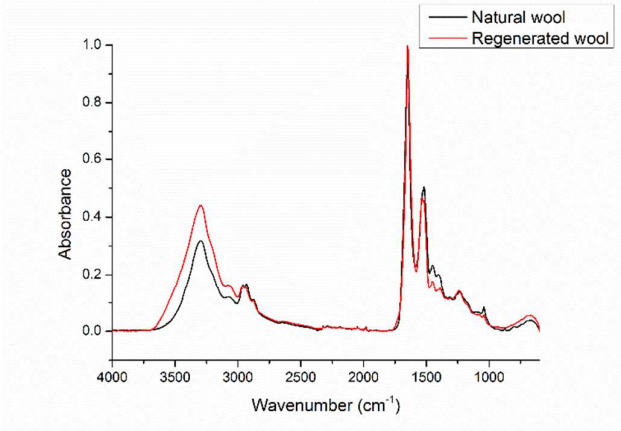
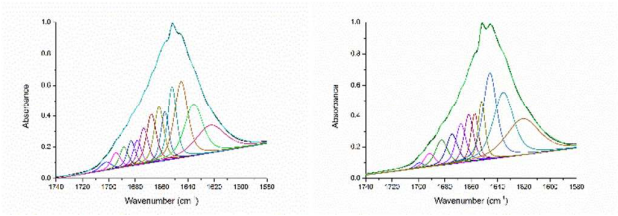


Fig. 4 ATR-FTIR of natural wool and regenerated wool.



(a) (b)

Fig. 5 FTIR peak resolution of Amide I spectral regions of natural wool (a) and regenerated wool (b), 1740–1580 cm^{-1}.

The Amide I band vibrational frequency can be used to determine the secondary structure of proteins because it is particularly sensitive to protein secondary structure. The Amide I mode was resolved in Gauss shaped bands corresponding to α-helix (1650 cm^{-1}), β-sheet (1620 cm^{-1}) and disordered structures (Fig. 5). The fitting was done between 1740 and 1580 cm^{-1}. On the basis of literature data, the absorption in the range of 1657–1651 cm^{-1} suggests the presence of the α-helix structure, whereas the bands related to the β-sheet structure fall in the 1631–1621 cm^{-1} range. The peaks at low intensity in the 1697–1670 cm^{-1} range indicate disordered keratin conformations.[14,16] As can be seen in Fig. 6, compared with the natural wool, regenerated wool show a decrease of the band area related to α-helix structure accompanied by an increase β-sheet of the band area. This suggests that the dissolving process destabilize the α-helix structure.

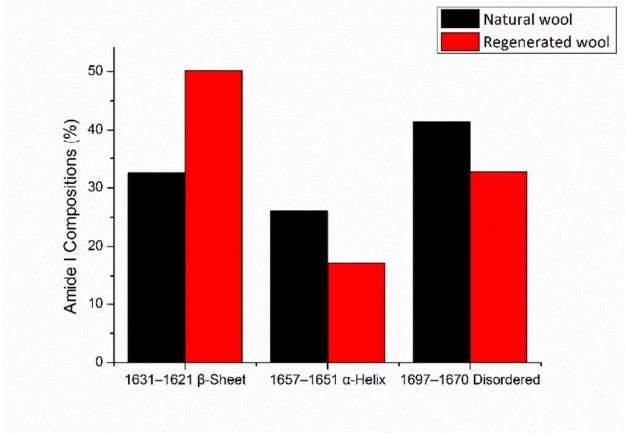


Fig. 6 Histogram illustrating curve-fitting results.

Raman studies. Fig. 7 shows the normalized Raman spectra of natural wool and regenerated wool. Normalization was carried out based on the band at 1450 cm^{-1} assigned to CH_2 bending mode, the peak area of which is large and not influenced by the extraction process. The bands lie in the wavenumber range of 500–1800 cm^{-1}, which are vibrations related to the S–S and C–S bonds of cysteine, amino acids like tryptophan, tyrosine, and phenylalanine; the Amide I and Amide III vibrations; and the C–C skeletal stretching vibration of the α-helix.[17] Peaks at 510±5, 525±5, and 540±5cm^{-1} are assigned to S–S stretching vibrations of gauche-gauche-gauche (g-g-g), gauche-gauchetrans (g-g-t), and trans-gauche-trans (t-g-t) conformers, respectively.[18,19] The bands recorded at 755 and 879 cm^{-1} can be assigned to tryptophan, while bands 852 and 1174 cm^{-1} can be ascribed to tyrosine. The medium strong bands at 1002 and 1035 cm^{-1} from phenylalanine can be ascribed to symmetric ring stretching and a C-H ring deformation mode. The peak at 1243 cm^{-1} is ascribed to the presence of randomly oriented structures of Amide III. A strong band is observed at 1450 cm^{-1} and assigned to CH_2 deformation of proteins and lipids. Tryptophan, another aromatic amino acid, exhibits a characteristic Raman band at 1551 cm^{-1}. The peak at 1614 cm^{-1} is assigned to C=C stretch vibrations of tryptophan, tyrosine, and phenylalanine.[17,18] According to the studies by Kuzuhara,[17] ratio of peak area of S-S bands (calculated from the peak to a baseline that was drawn between 485 and 570 cm^{-1}) to the peak area of the CH_2 band (calculated from the peak to a baseline that was drawn between 1428 and 1500 cm^{-1}) of Raman spectrum described the content of S-S in wool keratin. The calculated ratio of peak areas of S–S bands is 0.29 for natural wool and is 0.11 for regenerated wool, indicating that the content of S–S crosslinkages for regenerated wool keratin significantly decreased and broke about 62% of the S–S crosslinkages in the natural wool. Compared with natural wool, Raman spectrum of wool keratin has lower intensities at 932 cm^{-1} (marker for α-helix, C–C skeletal stretching of peptide backbone), 1317 cm^{-1} (C–H bending of α-helix) and 1655 cm^{-1} (α-helix in Amide I), which is indicated the decreasing of α-helix.

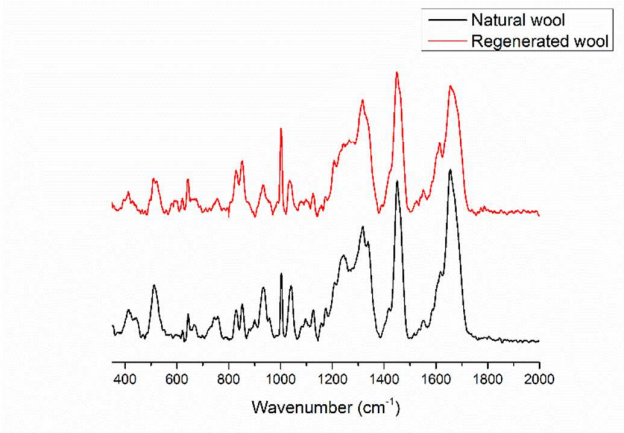
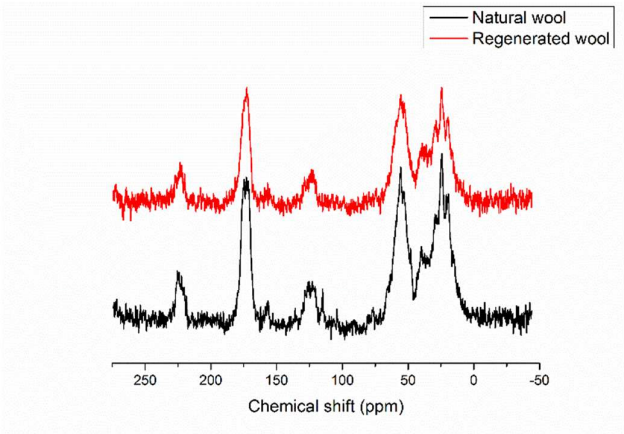


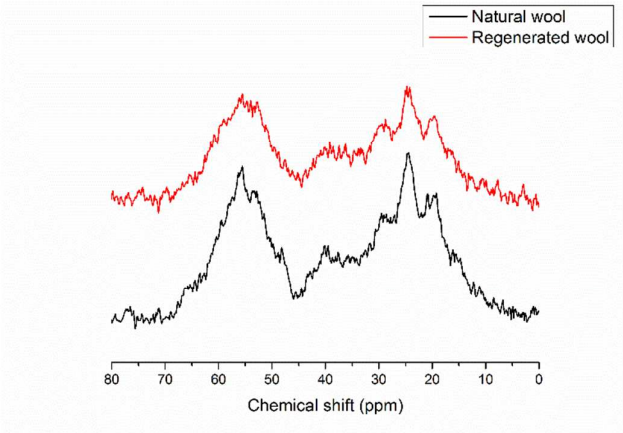
Fig. 7 Normalized Raman spectra of the natural wool and regenerated wool materials.

Solid state NMR studies. ^{13}C CP MAS spectra of the raw and regenerated keratin materials are shown in Fig. 8. The spectra show an asymmetric peak at 173.8 ppm, which is assigned to the amide carbonyl carbons of the keratin protein. The peak at 129.9 ppm is related to the aromatic group containing amino acids in the keratin. The α-carbons are recorded between 52 ppm and 56 ppm, while the β-carbons in leucine residues and cross-linked cysteine residues are observed at 40.1 ppm. The carbon peak recorded at 17.5 ppm can be assigned to alanine, while the peak at 22.4 ppm of natural wool is due to β-carbons of leucine. The intense peak centred at 25.0 ppm can be ascribed to the presence of β-carbons in glutamic acid, glutamine residues, arginine and cysteine. The NMR signal at low chemical shifts is associated with the alkyl groups of the side chains. [10,20-22]

The α-carbon peak between 52 ppm and 56 ppm is broadened in the regenerated keratin materials. The broadening of peaks in the regenerated keratin samples may be due to the ability of the L-cysteine to disrupt the hydrogen bonding of the original keratin raw material, leading to the unfolding of the polypeptide chains.[10] This would result in the formation of a greater fraction of β-sheet structures, in agreement with the XRD data discussed above.



(a)



(b)

Fig. 8 The ^{13}C CP MAS NMR spectra of the natural wool and regenerated wool materials (a); Enlarged version of the ^{13}C CP MAS NMR spectra (b).

Thermal stability. The thermal stability of the materials was investigated by Thermogravimetric Analysis (TGA), (Fig. 9). A two-step decomposition process was observed in all cases. The weight loss of natural wool keratin fibres and regenerated wool keratin were about 8% when temperature was heated to 100 °C that could be attributed to the loss of water. About 60% of the weight loss from 200 °C to 450 °C was mainly caused by the destruction of lateral chain of wool protein molecules.[10,23,24] The volatile compounds including hydrogen sulphide and sulphur dioxide are released from wool due to the cleavage of the disulfide bonds that occurred between 230°C and 250°C.[10] Fig 10 shows that maximum temperature of thermal decomposition of regenerated wool keratin was lower compared to the natural wool, probably due to the cleavage of the disulfide bonds during the extraction process of regenerated wool keratin.[23,24]

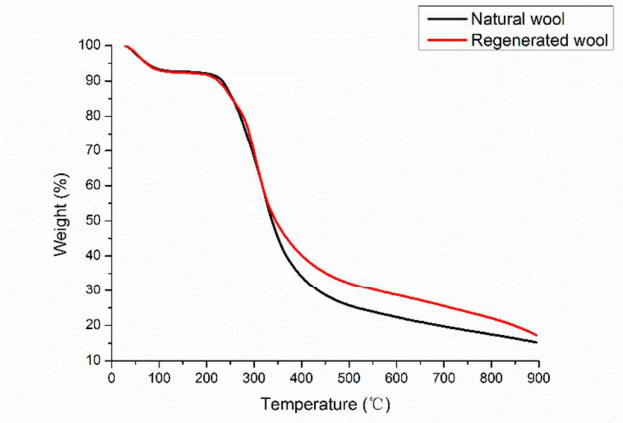


Fig. 9 TGA plots of the natural wool and regenerated wool materials.

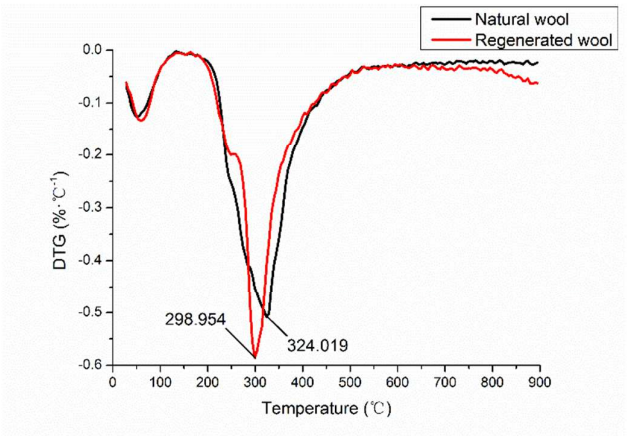


Fig. 10 DTG plots of wool keratin fibres and regenerated wool keratin**.**

Conclusions

In this study, L-cysteine was applied to the dissolution of wool keratin for the first time as a reducing agent. The dissolution time was 5h at 75°C, with the 72% dissolubility. It can be seen from the results that L-cysteine is an excellent reducing agent for extracting wool keratin. Compared with the structure of natural wool keratin fibres, the regenerated wool keratin from L-cysteine exhibits an increase of the β-sheet structure with the decrease of the α-helix structure, as observed from XRD, ATR-FTIR and ^{13}C NMR. It can be calculated from the Raman spectra the content of S–S crosslinkages for regenerated wool keratin significantly decreased and broke about 62% of those in the natural wool. Due to the cleavage of the disulfide bonds during the extraction process, the maximum temperature of thermal decomposition of regenerated wool keratin was lower compared to the natural wool.

Acknowledgements

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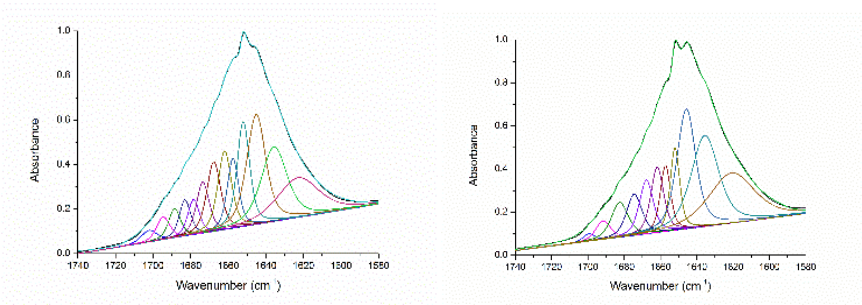
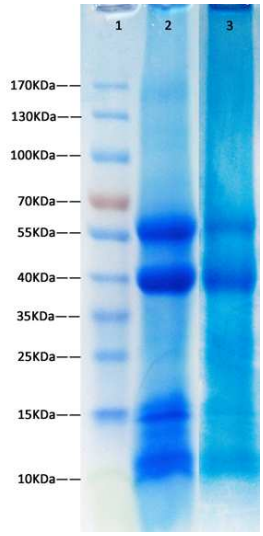
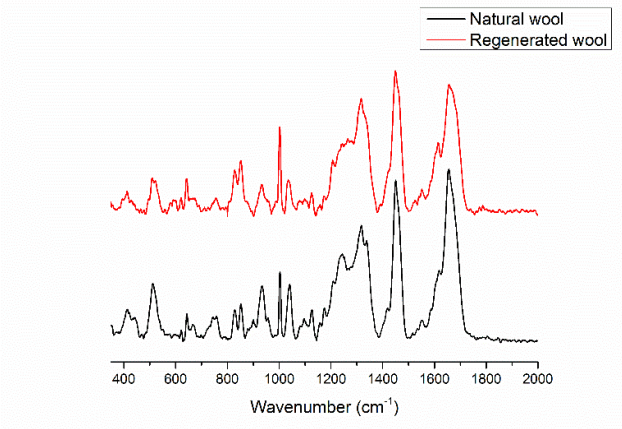


(a) (b)

FTIR Peak Resolution of Amide I spectral regions of natural wool (a) and regenerated wool (b), 1740–1580 cm^{-1}.



SDS-PAGE pattern of (1) protein standard (Thermo Scientific, MW 10,000-170,000) (2) natural wool (3) regenerated wool.



Normalized Raman spectra of the natural wool and regenerated wool materials

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