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COMMUNICATION

A sustainable solvent based on lactic acid and L-cysteine for the regeneration of keratin from waste wool

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Keratin dissolution is the first step toward reusing protein-rich biomass such as waste wool, hair, and feather. This paper reports an efficient and environmentally friendly method for the complete recycling of waste wool using a mixture of lactic acid and L-cysteine as a new green deep eutectic solvent (DES) for isolation of keratin. The dissolution time for a 90% dissolubility was 3.5 hours at 95 °C where 22 mg of wool was dissolved per 1 g of the DES. Keratin was obtained after dialysis of the DES mixture followed by lyophilization. In comparison with the raw wool, the α-helix content of keratin decreased while its β content increased. The keratin isolation with the proposed DES only requires water, L-cysteine, and lactic acid and does not require conventional chemicals such as urea, sodium sulfite, and sodium hydroxide.

One of the most abundant sources of keratin is wool, a by-product of the textile industry that consists of 95 wt% keratin. Approximately, more than 10 000 tons of waste wool is produced annually.[1] Keratin has intriguing characteristics such as biocompatibility, biodegradability, self-assembly, durability, and non-toxicity[2,3] and therefore it has a wide range of value-added applications in the fields of biomedicine, food, agriculture, textile, and cosmetics.[4] Developing a green and efficient method for the isolation of keratin from keratin-rich waste biomass has become a research hotspot which can simultaneously mitigate the environmental issues of waste streams.[5,6] Keratin is a fibrous protein rich in cysteine content (11–17 wt%).[2,7] Cysteine is a hydrophobic aliphatic amino acid containing a thiol functional group (–SH) ionizable at physiological pH (pH ~ 7.00) with the ability to form a disulfide bridge (Cys-S–S-Cys) in its oxidized form. High-density disulfide cross linkages with the highest bond energy have connected different zones of the polypeptide chains of the keratin molecule and along with the hydrophobic interactions and van der Waals forces they endow the keratin with high mechanical strength and structural stability.[8]

Various methods for keratin dissolution and extraction have been applied; however, the available processes have shown some shortcomings and disadvantages.[9] Utilization of concentrated strong acids or bases in chemical methods damages the protein structure by dissociation of peptide bonds, consequently decreasing product yields. Also, thiols that are conventionally used in this context may be harmful, usually toxic, and hard to work with,[10] limiting their commercialization and the scaling up of the used methods.[9]

Furthermore, a significant reduction in the cystine content of the final keratin and loss of water-soluble amino acids in thermal approaches, such as steam flash explosion and microwave, limit their applicability in keratin extraction from natural wool.[11]

With the development of green chemistry, ionic liquids and deep eutectic solvents, as green and designable solvents, have received much attention in protein-rich biomass solubilization and regeneration. In 2005, a 1-butyl-3-methylimidazolium chloride ionic liquid (IL) was used for the dissolution and regeneration of wool keratin fibers[12]; however, only 4 wt% of wool dissolved after 10 hours of treatment with the ionic liquid at 100 °C and under N_2.

Later in 2014, wool was dissolved in a 1-allyl-3-methylimidazolium dicyanamide [AMIM][dca] ionic liquid; however, dissolution was achieved at a very high temperature of 130 °C.[13] The high processing temperature can damage the protein structure and make the regenerated keratin unsuitable for many applications of biomaterials. Besides, the biodegradability and non-toxicity of these IL chemicals remain unanswered.

The potential of utilization of deep eutectic solvents (DES) which are formed by adequate mixtures of hydrogen bond acceptors and hydrogen bond donors has been demonstrated in keratin extraction as a simple, green, cost-effective and more efficient approach compared to common chemical and thermal approaches.[5]

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To date, the literature has also shown many examples of utilizing DES including choline chloride/urea[14,15] and choline chloride/oxalic acid[6] for the extraction of keratin from wools and feathers. However, the DES normally requires a high temperature (170 °C)[15] and results in low molecular weight keratin.[6]

In 2016, L-cysteine as a green reducing agent was first applied in the dissolution of wool keratin. Nevertheless, to achieve the dissolution of wool, L-cysteine was added to a concentrated solution of urea (8 M), with the pH adjusted to >10 using NaOH, and the dissolubility of 72% was achieved after 5 h of heating at 75 °C.[16]

In this present study, a new DES was prepared using a mixture of L-cysteine and lactic acid which was employed for the first time to dissolve wool and regenerate keratin. The structure and characteristics of the extracted keratin were investigated by SDS gel electrophoresis, Fourier transform infrared (FTIR) spectroscopy, thermogravimetry (TGA), differential scanning calorimetry (DSC), and X-ray powder diffraction (XRD).

The wool was defatted by Soxhlet extraction (1 : 1, v/v hexane/dichloromethane) for 16 hours, rinsed with distilled water, dried in an oven at 60 °C, and then cut into snippets prior to the experiment. Then, 0.4 g of the dried and defatted wool was treated with the DES solution composed of 2 g L-cysteine (Merck, Darmstadt, Germany) dissolved in 20 mL of L(+)-lactic acid (90% solution in water) (Fisher Scientific Merelbeke, Belgium) for 3.5 hours at 95 °C in a closed lab bottle. The mixture was sieved and then dialyzed in distilled water using a 3500 Da membrane for 3 days at room temperature and the water for dialysis was replaced 3 times per day. To recover the keratin, the mixture was dried in a freeze dryer and finally the keratin powder was sealed and stored at 4 °C before characterization experiments.

The solvent destabilizes the macromolecular structure by weakening the intermolecular and intramolecular forces, and breaking down the disulfide bonds has the potential to dissolve and extract keratin from wool without damaging the peptide bonds.[6,8] The DES in this study is formed by the combination of L-cysteine (amino acid) and L(+)-lactic acid (2 : 20, w/v). Lactic acid (2-hydroxypropanoic acid) is a carboxylic acid with a pK_a value of 3.86 (20 °C) and is a natural hydrogen bond donor (HBD).[17] It can be naturally produced by bacterial fermentation of sugar substrates. Compared to mineral acids, carboxylic acids such as lactic acid are weak to protonate the carboxylate side chains in wool; however, they readily form hydrogen bonds with peptides and other functional groups in wool and in this way promote fiber swelling. Lactic acid has also been used for some skin treatment as it can markedly plasticize the outer layer of skin (stratum corneum), which consists of keratinised cells. Lactic acid solvent has demonstrated many advantages such as its bio-based origin, superior synthetic efficiency, ease of isolating the product and good recyclability which make it a promising green extraction solvent and an effective means for designing environmentally benign synthetic systems.[18]

Lactic acid softens the stratum corneum by adsorbing to the polar groups of keratin chains and reducing interactions between them without increasing the water content in the stratum corneum. The value of lactic acid as a softening agent for keratin has been known for many years.[19–21]

The stiffness and strength of wool fibers are due to many intra- and intermolecular disulfide bonds because of a high content of cysteine residue in the backbone of the polypeptide.

When wool samples are heated in the presence of lactic acid, the compact crystalline structure of the keratin is denatured and it becomes amorphous and the wool softens and swells. This behavior facilitates the penetration of L-cysteine and exposure of disulfide bonds to L-cysteine which eventually reduce the disulfide bonds and result in the dissolution of wool (Fig. 1a). However, this is one suggested mechanism for the dissolution of wool in the DES (lactic acid/L-cysteine) and we will investigate this hypothesis in our future testing.

Lactic acid can solubilize L-cysteine and swell wool at the same time so there is no extra need to use a high concentration of urea compared to the method used by Wang *et al.*[3] in which L-cysteine with a high concentrated solution of urea (8 M) was used. In addition, we achieved a higher dissolution (90% compared to 72%) at a lower processing time of 3.5 hours. In addition, lactic acid is non-toxic with large availability on the market and it is mostly produced by fermenta-

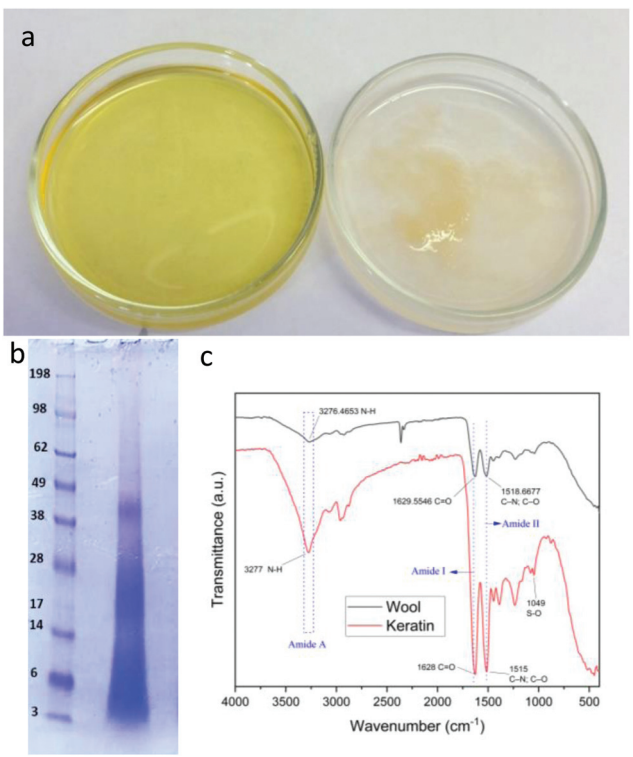

Fig. 1 (a) Wool sample before (right) and after (left) solubilisation in the deep eutectic solvent, (b) SDS-PAGE pattern of the protein standard (left) and the regenerated keratin (right), and (c) FTIR spectrum of natural wool and regenerated keratin.

tion of carbohydrates such as glucose, sucrose, or lactose.[22] The average molecular weight, pattern, and purity of extracted keratin using DES (lactic acid/L-cysteine) were investigated by SDS-PAGE gel electrophoresis and compared to standard markers (Fig. 1b). The regenerated keratin showed the characteristic band of wool protein between 38 and 49 kDa, two major bands between 14 and 28 kDa, and a lower molecular weight region of 3–6 kDa. SDS-PAGE results showed that the main structure of keratin was preserved. Both wool and keratin exhibited similar FTIR spectra without the generation of any new bands in the keratin spectra (Fig. 1c) indicating the same structure and functional groups of the samples. Wool and keratin samples showed the characteristic peaks of amide A at 3271 and 3278 cm^{-1}, respectively, which are attributed to the N–H stretching of a peptide bond. The characteristic band of amide I assigned to the C═O bond was observed at around 1628 cm^{-1} for wool and keratin. Besides, the characteristic peak of amide III observed around 1515–1518 cm^{-1} for the wool and regenerated keratin sample can be attributed to the C–N and C–O stretching vibrations.[6,16,23] Furthermore, the CH, CH_2, and CH_3 vibration peaks appeared between 2800 and 3000 cm^{-1} for both wool and keratin. The keratin sample exhibited a characteristic peak around 1049 cm^{-1} attributed to the symmetric and asymmetric S–O stretching vibrations, indicating the presence of cysteine–S–sulfonated residues in the extracted keratin.[24] The presence of a reduced form of (–SH–) keratin can be interpreted to be due to the presence of bands between 560 and 730 cm^{-1} in the keratin spectra.[2,25] Fig. 2a demonstrates the X-ray diffraction pattern of wool and extracted keratin. Both wool and keratin showed two characteristic peaks around 9° and 20.2° attributed to the typical diffraction pattern of α-keratin.[26] The α-helix structure shows two characteristics peaks at 2θ = 9° and 17.8°, while the β-sheet structure exhibits two peaks around 2θ = 9° and 20°.[27] Our results showed that the intensity of the peak at 9° significantly decreased after the dissolution due to the destruction of the α-helix structure by the L-cysteine in the dissolution process. Interestingly, the peak around 20° was stronger in the extracted keratin sample compared to that in natural wool, indicating the in the number of β-sheet structures in extracted keratin.

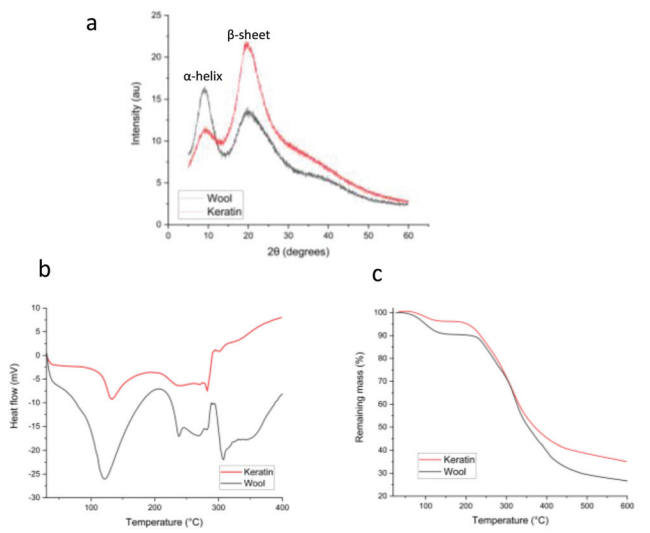

Fig. 2 (a) XRD pattern of natural wool and extracted keratin, (b) DSC thermograms in the 30–400 °C range of wool and keratin powder heated under a dynamic flow of nitrogen at a heating rate of 10 °C min^{-1}, and (c) TGA curves of wool and keratin heated from 30 to 600 °C at 10 °C min^{-1} under a dynamic nitrogen atmosphere.

Thermal properties of keratin were investigated using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The DSC thermogram of samples (Fig. 2b) showed an endothermic peak between 80 and 150 °C attributed to the evaporation of water from both wool and keratin samples.[2] Moreover, an exothermic peak around 230–260 °C was observed for both wool and keratin samples which is assigned to the thermal denaturation of the α-helical crystallites[27,28] and also corresponding to the degradation of peptide bonds.[29] However, the keratin sample exhibited a very broad peak around 240 °C possibly indicating the lower amount of α-helix structure due to the dissolution in L-cysteine, which is consistent with the XRD and FTIR results. Furthermore, a two-step decomposition was observed in the TGA curves of both wool and keratin (Fig. 2b). The first degradation step attributed to water loss occurred between 80 and 150 °C with mass loss values of 9.59 and 4.82% for wool and keratin, respectively. The second stage of decomposition occurred between 200 and 400 °C attributed to the degradation of keratin polypeptides comprising the decomposition of disulfide bonds resulting in the release of hydrogen sulfide and sulfur dioxide.[16,30–32] Natural wool showed a weight loss of 73.36% within the second stage of decomposition while the weight of keratin was 65.62%. The maximum degradation temperatures (DTG_{max}) of wool decreased after the dissolution in L-cysteine from 323.3 °C to 298.6 °C (keratin). The DSC and TGA results indicate that keratin showed a similar profile in comparison with the wool indicating that keratin could maintain its thermal and conformational stability after the dissolution in L-cysteine.

In this study, we have shown that the eutectic mixture of L-cysteine/lactic acid works as an excellent solvent for the dissolution of waste wool. Regenerated keratin from the DES exhibited a β-sheet structure and the disappearance of the α-helix and the regenerated keratin showed similar thermal stability to the natural wool. Although the process of keratin solubilization still needs to be optimized in terms of the processing parameters such as the solid to liquid ratio, processing temperature and time, our results demonstrate that the new DES based on L-cystine and lactic acid is an outstanding green candidate for the regeneration of wool keratin without losing the long peptide chains. The method also offered the possibility of reusing the large-scale waste wool.

Conflicts of interest

There are no conflicts to declare.

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