CONVERSION OF WASTE WOOL TO PROTEIN USING A SIMPLE OXIDATION METHOD

Amin Shavandi 1, Alan Carne 2, Adnan A. Bekhit 3, Alaa El-Din A. Bekhit 1*,

1 Department of Food Sciences, University of Otago, Dunedin, New Zealand
2 Department of Biochemistry, University of Otago, Dunedin, New Zealand
3 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt

ABSTRACT- Wool keratin is a potentially important natural source of renewable protein with many applications. Dissolution of keratin is the major challenge for utilization of wool waste. The yield of keratin from peracetic acid (PA) treatment was investigated using 6, 12, 24 and 36% PA for 1, 2, or 3 days of treatment time. PA at 24% and 2 days resulted in the generation of water soluble (WSK) (57%) and insoluble keratin (ISK) (40%) and was found to be the optimum processing condition. The physiochemical properties of the obtained samples were determined using SEM, XRD, FTIR, SDS-PAGE. Extracted keratin samples showed no toxicity to fibroblast (L929) cells up to a concentration of 2.5 mg/ml. Both WSK and ISK had a fibrous microscopic structure and when analysed by FTIR the extracts showed a high content of cysteine-S-sulfonated residues and SDS-PAGE confirmed that the extracts contained proteins in the 40-60 kDa molecular weight range.

1. Introduction

Keratin as the major component of wool, hair, nail, feather and horn, is one of the most abundant and under-exploited protein containing materials that has challenging disposal issues. More than 5 million tonnes of keratin-containing by-products are produced annually (Barone, Schmidt et al. 2005), but currently these by-products have limited applications such as use in fertilizer or in biodegradable surfactants. Disposal of keratin-containing materials by incineration can be environmentally problematic since they contain 3-4% sulfur (Zoccola, Aluigi et al. 2012) which can contribute to significant pollution. Wool consists of about 95% keratin proteins containing 5-10 mol % cysteine residues. Therefore, a large quantity of keratin protein rich biomass is under-utilized and so it is environmentally and economically important to transform wool into value added products. The main challenge to achieving this goal is to develop a simple and cheap method to extract keratin. Cystine has an important role in determining the physiochemical properties of wool keratin, and compared to most other proteins, keratin has higher stability and lower solubility due to inter- and intra-chain cross-links of cysteine disulfide bonds. Also hydrogen, hydrophobic and ionic bonds play an influential role in stability and properties of the wool keratin. The presence of ionic bonds is pH dependent and is highest at the isoelectric point of pH=4.9 when the protein is in the form of zwitterions (+H3N—CHR—COO—), while in extremely acidic or basic conditions is at the lowest level. Considering that ionic bonds occur between carboxylic anions and ammonium cations, these bonds can be disrupted by protonation of the carboxylic group at low pH and deprotonation of amine group at high pH (Tonin, Aluigi et al. 2010). Available literature on the extraction of keratin using the oxidation method has been mainly focussed on separation of keratin into α, β and γ forms and investigation of biomedical applications. The conditions used in the extraction of keratin using PA have been mild, using a low acid concentration (≥ 2%) and have been relatively similar over the years (Edwards, Jarvis et al. 2015). This has resulted in the solubilisation of 6% α keratin (Buchanan 1977) or a mixture of β and γ keratin from wool [14-16]. This keratin has been used in medical applications such as ocular and nerve regeneration, or drug delivery and tissue engineering (Srinivasan, Kumar et al. 2010). The aim of this study was to evaluate the extraction yield of keratin from wool at higher concentrations of PA (6, 12, 24 and 36% v/v) with longer extraction times (1, 2, and 3 days) and to investigate physicochemical properties of the keratin samples obtained with the extraction conditions.

2. MATERIALS AND METHODS

Waste New Zealand Merino wool fibres with a mean diameter of 21 µm, were obtained from the New Zealand Merino Company, Christchurch, New Zealand. Peracetic acid (PA) and Tris were obtained from Sigma. The wool was defatted by Soxhlet extraction. In order to optimize the concentration of PA and the time required for oxidation, known weights (5 g) of wool samples were treated with 330 ml of PA solution (6, 12, 24 and 36 % v/v) for 1, 2, and 3 days at room temperature. Then the wool samples were taken out at intervals of 1 day using a stainless steel mesh and rinsed with deionised water until the rinse solution was neutral pH. The wool was then treated with 100 mM Tris solution and shaken at 150 rpm using a rotary shaker (Ratek, Victoria, Australia) to extract the keratin. The mixture was then vacuum filtered which generated a thick jelled keratin retentate and a solubilized keratin filtrate fraction (Fig 1). Both fractions were then dialysed (12-14 kDa cut-off membrane) against 5 L distilled water changed 3 times a day for 3 days, followed by freeze-drying (Edwards, Jarvis et al. 2015). The two obtained powder fractions were then tested for solubility in water and named water soluble keratin (WSK), soluble in water, up to 10mg/ml and insoluble keratin (ISK) respectively. The keratin extraction experiments were repeated 3 times.

Keratin extraction yield and total protein yield

The extraction yield of keratin was calculated from the ratio of the weight of the freeze dried keratin extract (W”) and initial weight of wool (W”) using following equation

\[
\text{Ex.Yield} \% = \frac{W’}{W”}
\]
The total protein in each fraction was determined in triplicate using the Kjeldahl method (International 2005).

### Characterization of extracted keratin

The phase composition of the extracted keratin was determined using an X-ray diffractometer (XRD; PANalytical X’Pert PRO MPD System) in the range 20°<2θ<60° with Cu Kα radiation (k=0.15418 nm). Fourier transform infrared spectroscopy (ALPHA FT-IR; Bruker) was used for identification of the functional groups present in the keratin extracts. The FTIR spectra were obtained in ATR mode over the region 400–4,000 cm⁻¹ with a 4 cm⁻¹ spectral resolution (Aluigi, Tonetti et al. 2014). The thermal stability of the extracts was analysed using thermal gravimetric analysis (TGA; Q 500, TA instruments) from 20°C up to 1000°C at a heating rate of 10°C/min. The molecular weights of the solubilized keratin extracted proteins were determined using SDS-PAGE (Aluigi, Tonetti et al. 2014). Elemental analysis (C, H, N, and S) of the samples was determined using an elementary analyzer (Carlo Erba Elemental Analyser EA 1108). The elemental profile of the samples was measured using an inductively coupled plasma mass spectrometry system (ICP-MS; Agilent 7500cs). Solid state CP-MAS 13C NMR spectra were acquired at 50.3 MHz on a Bruker Avance III 200 spectrometer (Bruker, Switzerland).

### 3. RESULTS AND DISCUSSION

#### 3.1. Concentration of peracetic acid and treatment time

In this study, 24% (v/v) PA was used for the extraction of keratin from the wool. This acid concentration was selected based on results obtained using various acid concentrations (6, 12, 24 and 36% v/v) and treatment times (1, 2, and 3 days) as shown in Fig 1. Using 6% (v/v) PA resulted in 11.22 ± 1.66 % yield of WSK after 3 days of treatment time. The yield increased to 25.10 ± 2.85 % using 12% (v/v) acid for the same treatment time (3 days). With 24% (v/v) acid the extraction yield of WSK increased to 54.93 ± 3.53 % after 2 days of acid treatment. The extraction process was faster when 36% (v/v) acid was used and the WSK extraction yield reached 52.86 ± 6.03 % after 1 day of treatment. The yield obtained using 36% acid (52.86 ± 6.03 % after 1 day) was not significantly different (p > 0.05) from that using 24% acid (v/v) and 2 days treatment time (Fig. 2). Therefore, considering the lower use of acid, the 24% (v/v) extraction was selected for further studies (Fig. 2). The yields of keratin fractions (WSK and ISK) extracted by oxidation using 24% peracetic acid are summarized in Fig 2. Keratin extracts obtained from 1 day of treatment had the lowest amount of WSK and highest ISK while the highest WSK content was obtained after 2 and 3 days of treatment. There were no significant differences between the keratin extract yield (WSK or ISK) from day 2 or day 3. It was not easy to separate the ISK and WSK phases after 3 days, and the insoluble extract phase (ISK) was degraded to a viscous like mixture that was not separated easily using filtration or centrifugation. Therefore, the 24% peracetic acid and 2 days treatment time were selected for further studies.

#### 3.2. Dissolution of the wool

The wool samples did not appear to behave as a chemically homogeneous protein material (Earland and Knight 1955). Therefore, during the oxidation process, the bulk of the wool samples were solubilized but there was some insoluble material in all of the treated wool samples which is believed to be mainly from the cuticle and mainly composed of β- keratin (Timmons, Blanchard et al. 2003). The keratin extract solution mainly consisted of α-keratin which is predominantly found in the cortex and has a crystalline structure. The keratin residue in the form of a thick jelled material is mainly β-keratin which is primarily found in the wool cuticle. It has been reported that the folded α-form of keratin is more soluble than the extended β from (Earland and Knight 1955). Treatment with PA partially oxidizes the naturally occurring disulfide linkages of keratin and converts them to hydrophilic pendant sulfonic groups on the side chains of the cysteine amino acids that can complex with water. In this study, the high concentration of PA (24%), compared to the 2% used in previous studies (Buchanan 1977), provided a low pH that resulted in partial breakage of hydrogen bonds that makes keratin soluble. Therefore, a higher yield of soluble keratin was obtained in this report as a result of a synergistic effect of low pH breakage of hydrogen bonds and disruption of the disulfide bonds during the oxidation process. Similar to the sulfonic groups that are hydrophilic, breakage of hydrogen bonds also causes swelling and hydration of the keratin proteins (Greenberg and Fudge 2013). The ISK can absorb water and swell and forms a hydrogel but is not soluble at neutral pH; however, using lyophilisation the obtained dried material was found to be highly hygroscopic. The obtained ISK and WSK, therefore, appear to have a different configuration, composition and chemical structures.

#### 3.3. Characterization of the keratin samples

**IR spectra**

The IR spectra of natural wool, WSK and ISK are shown in Fig 3 (A1-A3). The FTIR spectra showed the absorption bands of the peptide bonds (-CONH-). The band at around 3276 cm⁻¹ is attributed to amide A, which is connected with N-H stretching bonds. The C=O stretching vibrations that provide a connection for amide I can be observed in the range of 1700-1600 cm⁻¹. The band at 1518 cm⁻¹ is related to amide II that has N-H bending and C-H stretching vibration bands, and amide III bands can be seen at 1200-1300 cm⁻¹ which are related to C-N, C-C stretching and N-H, C-O bending (Aluigi, Zoccola et al. 2007, Wang, Li et al. 2015). The expanded area of 1500-1700 cm⁻¹ (Fig 3. A3) shows a sharp band of the α-helix structure at 1650-1657 cm⁻¹ that belongs to amide I and indicates the significant portion of the α-helix structure. The bands attributed to β-sheet can be observed in 1620-1630 cm⁻¹ and region of below 1550 cm⁻¹ (Aluigi, Zoccola et al. 2007). Wool oxidation resulted in the appearance of new bands at around 1040 (stretching mode of S-O sulfonate), 1120 (S-O, cysteine dioxime) and 1175 cm⁻¹ (sulfonate, S-O stretching) which is in agreement with previous works (Robbins 1967, Alter and Bit-Alkhas 1969). The bands at 1140-1180 cm⁻¹ and 1300-1350 cm⁻¹ are attributed to the sulfonamide type of grouping -SO₂. New bands appeared at 515, 580 cm⁻¹ corresponding to S-O linkage of sulfonate and bands at 810, 740 cm⁻¹ that are attributed to stretching mode of C-S sulfonate. The bands observed below 900 cm⁻¹ correspond to cysteic acid and sodium octanesulfonate (After
and Bit-Alkhas 1969). The bands at 1040 and 1075 cm$^{-1}$ observed in the untreated wool sample indicate to cysteic acid and sulfonyl residues, which might be a result of air oxidation of keratin present in the cuticles (Robbins 1967). It has been proposed that the bands appearing at 1150-1210 cm$^{-1}$ and 1030-1060 cm$^{-1}$ are corresponding to the sulfonic acid group. The weak band of ISK might indicate a high proportion of intact and unfolded keratin structure (Ghosh, Clerens et al. 2014) in this keratin fraction.

**X-ray diffraction analysis**
The XRD patterns of WSK and ISK in Fig 4 show two prominent peaks at about 20=9° and 19.6°. The peak at about 9° corresponds to both α-helix and β-sheet crystalline structures and shows that α-helix was preserved during the extraction process and was not destroyed as found with other extraction methods such as ionic liquids, for which a disappearance of peaks at about 10° was reported (Li and Wang 2013). The broad peak at about 20=21° is probably due to overlapping of the α-helix peak at about 17.8° and the typical β-sheet structure peak at about 19°. However, the discretion of peaks was not possible due to overlapping of the peaks (Aluigi, Zoccola et al. 2007). It can be seen that both samples had similar diffraction patterns, nevertheless, the peaks at about 9° and 21° are stronger and sharper in the WSK sample, suggesting a higher content of β-sheet structure (Idris, Vijayaraghavan et al. 2013, Li and Wang 2013).

**Thermal stability of the keratin samples (TGA and DSC)**
Both samples recorded a two-stage weight loss (Fig 5A). The weight loss from 200-400°C is attributed to the lateral chain destruction of wool proteins. Hydrogen sulfide and sulfur dioxide are released in this heating stage as a result of breakage of disulfide bonds at 230-250°C. As shown in the figure, ISK had higher thermal stability compared to WSK. In fact, ISK also showed higher thermal stability for the whole range of tested temperatures. This high stability can be due to a higher proportion of unfolded structure and higher crystallinity of the ISK samples compared to WSK. Also, the better thermal resistance of ISK can be attributed to the higher amount of β-sheet structure, which has chains with stronger intermolecular interactions. The destruction of the wool protein lateral chain might be the main reason for the weight loss of the samples during the temperature transition from 150 to 500°C (Li and Wang 2013). The DSC thermogram of the samples is shown in Fig 4B. The endotherm peak observed at around 100°C is related to the evaporation of water (Aluigi, Zoccola et al. 2007). The second endotherm peak at around 220°C is related to the denaturation of α-helix structure, referred to as the crystalline melting peak (Barone and Schmidt 2006) which occurs at lower temperature for WSK (212°C) compared to ISK (223°C). The results observed in this study, are similar to that reported by Wang et al. (Wang, Li et al. 2015).

**Molecular weight distribution**
Gel electrophoresis analysis was carried out to characterise and identify protein in the obtained WSK and ISK fractions (Fig 6). Both ISK and WSK showed a high intensity band between 100 to 30 kDa and between 45-58 kDa, however, the majority of the proteins had molecular weights (MW) higher than 40 kDa. The characteristic bands related to α-helical keratin proteins can be observed at 40 kDa and 64 kDa (Hill, Brantley et al. 2010). Molecular weight analysis of the ISK and WSK samples showed characteristic bands similar to that previously reported (Hill, Brantley et al. 2010), although in that study thioglycolic acid at pH 10.2 was used for extraction of keratin. It is generally suggested that keratin protein bands at a MW range of 11-28 kDa contain high sulfur whereas those at higher MW are low in sulfur (Zoccola, Aluigi et al. 2012, Ghosh, Clerens et al. 2014). No bands were observed in both samples for the high sulfur proteins of the matrix (11-28 kDa) which indicate that these proteins were not generated, while the low sulfur content proteins were remained intact (40-64 kDa) (Zoccola, Aluigi et al. 2012, Ghosh, Clerens et al. 2014). The presence of high molecular weight keratin indicates that the chemical structure of the keratin was not significantly affected by the oxidation extraction conditions (Zoccola, Aluigi et al. 2012).

**Solid-state NMR**
Both spectra in Fig 7A show an asymmetric distinct signal with maxima centred between 170 and 174 ppm, which indicate the carbonyl carbons in keratin (Duer, McDougall et al. 2003, Idris, Vijayaraghavan et al. 2013). The broad signal at 54 ppm is due to the α carbon and the signal at 40 ppm is due to the β carbon present in leucine residues and crosslinked cysteine (Duer, McDougall et al. 2003, Idris, Vijayaraghavan et al. 2013). The peaks at 54 ppm and 40 ppm are sharper in WSK samples, which indicate a more open structure for WSK compared to ISK. The complex signals at the low chemical shift region of 10-60 ppm are due to the presence of individual amino acid residues in the keratin protein and belong to alkyl side chains (Nishikawa, Tanizawa et al. 1998). Differences between ISK and WSK can be observed with shoulders at 17 ppm and 22 ppm, which can be due to the methyl group side chain of alanine and the β carbon of leucine, respectively. The signals at 115-160 ppm with maxima at 123 ppm belong to aromatic groups (Nishikawa, Tanizawa et al. 1998). WSK shows increased peaks due to aromatic groups at 110, 123 and 152 ppm compared to ISK. A similar pattern was reported by Carr and Geramimow (Carr and Geramimowicz 1988), who evaluated the effect of heating on wool and an increase in the aromatic group peaks was observed on increasing the temperature to 225°C. Both WSK and ISK also showed clear differences in the 40-65 ppm region, that is probably due to disruption and cleavage of the disulfide linkages (Idris, Vijayaraghavan et al. 2013). The secondary structure of the keratin isolates was investigated by deconvolution of the carbonyl peaks using the Gaussian fitting function (Fig 7B). There are two major peaks in the deconvoluted form, one attributed to the α-helix at around 175 ppm and another peak at 172 ppm which is related to β-sheet and random coil (Duer, McDougal et al. 2003, Idris, Vijayaraghavan et al. 2013). Nishikawa et al (Nishikawa, Tanizawa et al. 1998) suggested that the structural changes during the oxidation treatment resulted in the conversion of the α helix to random coil structure. The minor peak at 180 ppm, is ascribed to be the side chain of Glu, Asp and carboxymethyl-L-cysteine (Duer, McDougal et al. 2003). The differences observed in the intensity of the line shape of carbonyl I in the ISK and WSK samples can be due to a change in conformation of the keratin molecules (Duer, McDougal et al. 2003).
4. Conclusion

The physicochemical properties of keratin extracted from wool using 24% (v/v) peracetic acid were evaluated in this study. The extent of solubilization of keratin from wool is dependent on the concentration of peracetic acid used and the time duration of the process. The extraction process resulted in two keratin fractions, water soluble (WSK) and an insoluble (ISK) fraction. Both fractions retained the keratin backbone structure but breakage of hydrogen and disulfide bonds occurred. The best yield of the WSK fraction was 64% and was obtained after 3 days treatment of the wool. Using ATR-FTIR, XRD and NMR, it was observed that the WSK and ISK extracts did not have major chemical differences. However, the ISK contained a higher concentration of chemical elements, while the WSK showed weaker diffuse protein bands on SDS-PAGE and XRD pattern. The results obtained in this study indicate considerable potential for use of the keratin extracts. We are currently evaluating the mechanical properties of the keratin samples to evaluate their possible use for the production of fibre, film and injectable gel for biomedical applications.

References


Fig 1. Schematic of different chemical bonds present in keratin structure (a) and (b) schematic of the extraction of keratin from wool using a peracetic acid oxidation method.

Fig 2.a. Effect of different peracetic acid concentrations and treatment times on the extraction yield of water soluble keratin (WSK) from wool. The bars with different symbols (+, #, *) are significantly different ($p < 0.05$). The values are mean ± SD (n = 3). b. Different amounts of insoluble keratin (ISK) and soluble keratin (WSK) fractions obtained from wool samples treated with peracetic acid (24% v/v) for 1, 2 or 3 days. The amount of ISK and WSK obtained after day 1 is significantly different (*) ($p < 0.05$) from the WSK and ISK obtained from the day 2 and day 3 treatments. The values are mean ± SD (n = 3).

Fig 3. IR spectra of wool, water insoluble (ISK) and soluble keratin (WSK) (a1), the region of interest was expanded in Fig a2 and a3. Oxidation of the keratin results in transformation of disulfide to sulfonate (b).
Fig 4. The XRD patterns of water soluble (WSK) and insoluble (ISK) keratin extracts.

Fig 5a. The TGA and DSC thermograms of the soluble (WSK) and insoluble (ISK) keratin extracts. b. SDS-PAGE of water soluble (WSK) and insoluble keratin (ISK).

Fig 6a. 13C CP MAS NMR spectra of water insoluble keratin (ISK) and water soluble keratin (WSK) extracted from wool. The data were fitted with Gaussian/Lorentzian fitting functions. b. 13C CP MAS NMR spectra of water insoluble keratin (ISK) and water soluble keratin (WSK) regenerated from wool. The data were fitted with Gaussian/Lorentzian fitting functions.