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Comparison of Methods for Extraction of Keratin from Waste Wool

Eleanor M. Brown*, Kalgi Pandya, Maryann M. Taylor, Cheng-Kung Liu

Biobased and Other Animal Co-Products Research Unit, Eastern Regional Research Center, Agricultural Research Service, US Department of Agriculture, Wyndmoor, PA, USA
Email: *ellie.brown@ars.usda.gov

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Abstract

The U.S. sheep industry, more than 80,000 producers of 40 million pounds of raw wool per year, is an important component of the meat industry. New methods for the treatment of domestic wool with keratin isolated from the unmarketable fraction of wool, and functionalized for water, oil, or insect repellency are needed. As a first step in the process, we are evaluating the effectiveness of keratin solubilization via relatively benign methods that use thioglycolic acid, bisulfite or sulfide to reduce disulfide bonds, peracetic acid or percarbonate to oxidize disulfides, and urea/thiourea as hydrogen bond disrupters. The procedures are compared in terms of quality of soluble protein, cost effectiveness, potential for upscaling, environmental and operator safety. Successful completion of this project will provide the basis for commercial development of such methods, followed by functional modification of the soluble keratin, and its application to textiles.

Keywords

Waste Wool, Keratin Extraction, Economic Comparison

1. Introduction

The US sheep industry, a small but important component of the meat industry, consists of 80,000 producers of 5 million sheep and 27 million pounds of raw wool per year. Although domestic wool has properties that limit its acceptance and competitiveness when compared to imported wool, the military and many law enforcement agencies are required to use domestically raised and processed wool for their uniforms and other wool containing products. An enzymatically-modified wool, developed at this Center, in an earlier project has been evaluated by the US military for use in undergarments [1] [2]. Meeting additional preferences of military and general consumer markets for al-

tered wool properties—flame resistance, navy whiteness, and oil and water repellency—requires further research on the functional modification of woolen textiles and yarn.

Previous research from this laboratory has modified collagenous waste from the production of leather for utilization in leather finishing steps to improve the final product [3]-[6]. In a similar fashion, we propose to expand on the work of Gembeh *et al.* [7] and develop methods for modifying the wool protein, keratin, from the unmarketable fraction of domestic wool by adding characteristics such as water, oil, or insect repellency to the extracted protein and then incorporating the modified protein into wool textiles.

In contrast with collagen, where the fibrous proteins are distinct and separable, keratin is a heavily crosslinked network of proteinaceous material with variable composition. The recent publication by Deb-Choudhury *et al.* [8] is a comprehensive review of options for extracting keratins and keratin associated proteins from wool and hair. The past decade has seen an upsurge in research into utilization of waste keratin as a biotechnology resource. Having a soluble or at least semi-soluble substrate is essential for most protein modification processes, and the highly disulfide crosslinked structure of keratin presents a challenge. A variety of methods for preparing a soluble keratin fraction from human hair as well as from meat industry byproducts, mainly wool, hair, and feathers are available in the literature. The intent of this study is to evaluate some of these methods in the search for an effective, reasonably safe, environmentally responsible and cost effective method for preparing soluble keratin for future product development that would be expected to encourage domestic processing and open new markets for US wool.

2. Experimental

2.1. Materials

Wool fiber, 24.0 - 25.0 micron, scoured and combed, was supplied by Chargeurs Wool (USA) Jamestown, SC and analyzed for moisture, ash and residual lipid as described by Taylor *et al.* [9]. Reagent grade chemicals were used except where otherwise noted.

2.2. Removal of Residual Lipid

Wool fibers were cut into ~1/4 in lengths and treated by one of the following published procedures to remove residual lipid. 1) Washed for 2 h in a 0.1% solution of TWEEN 20, rinsed with water, and vacuum filtered on Whatman #2 paper [10]; 2) Immersed in a mixture of chloroform/methanol (2:1, v/v) for 24 h, and filtered as above [11]; 3) Soxhlet extracted with petroleum ether for 6 h [12] or 4) Soxhlet extracted with hexane for 6 h. After the extraction, fibers were air-dried, and stored under vacuum without heat overnight before weighing. Solvents were evaporated to dryness, and the residual lipid weighed.

2.3. Analysis of Wool for Moisture, Ash, and Residual Lipid

Moisture and ash were determined on ~0.5 g samples of wool before and after hexane

extraction as described by Taylor *et al.* [9]. For moisture determination, samples in tared weighing bottles were dried under vacuum at 50˚C for 48 h, then cooled in a desiccator and weighed. Dried samples from the moisture determination were transferred to porcelain dishes and ashed at 600˚C in a muffle furnace for 2 h, then cooled and weighed. Lipid determination for the wool prior to Soxhlet extraction with hexane was by chloroform extraction of wool that had been hydrolyzed in 6 M HCl [9]. For hexane-extracted wool, the lipid containing extraction solvent was transferred to weighed crystallizing dishes that were reweighed after the solvent was evaporated to dryness.

2.4. Solubilization

Initial solubilization experiments were carried out on 0.5 g to 2.0 g batches of extracted wool fibers. For the final comparison 1.0 g samples were used for oxidation and reduction methods not involving urea. The sample size for methods that used 5 M to 8 M urea was 0.5 g, because of the difficulty and expense of using large quantities of urea. Vacuum filtration on qualitative filter paper removed any remaining fibers after initial steps in solubilization. Final separations of soluble and insoluble extracted fractions were by centrifugation at 15,000 g for 30 min at ambient temperature. Supernatant and solid fractions from centrifugation were dialyzed in 6000 - 8000 MWCO (molecular weight cut off) tubing over 3 days with 6 changes of deionized water, and then lyophilized. All procedures were performed at least three times.

2.4.1. Oxidation—Peracetic Acid (PAA)

The method of Sando *et al.* [13] for isolation of keratin from wool was adapted as follows. Fibers were oxidized overnight at 37˚C and 180 rpm in 2% peracetic acid, then filtered and washed with water. The oxidized fibers were then extracted 2× with 100 mM Tris-base (pH 10.5) at 37˚C and 180 rpm for 3 h, and 1× with water. Extracts were pooled and adjusted to pH 4 with 0.5 M HCl to precipitate protein. Precipitated protein was allowed to settle overnight. The liquid phase was decanted, and the precipitate was washed with 100 mM Tris-HCl pH 4 and then redissolved in 100 mM Tris-base, pH ~ 7.5.

2.4.2. Oxidation—Percarbonate (PCC)

A second oxidation method was based on the leather related oxidative dehairing studies of Shi *et al.* [14] and Marmer and Dudley [15]. A 30% solution of NaOH in water was prepared and allowed to cool overnight. Wool fibers were hydrated by soaking overnight in water. On the next day, excess water was removed by filtration, and the cooled NaOH solution was added at 3% on the weight of dry wool. After the wet wool and NaOH were thoroughly mixed, solid sodium percarbonate was added at 4.5% on the weight of dry wool. The mixture was stirred for 3 to 4 h until wool fibers were no longer visible.

2.4.3. Reduction/Oxidation—Thioglycolate/Hydrogen Peroxide (TGA)

The reduction/oxidation method of Hatakeyama *et al.* [10] for extracting keratin from

human hair was adapted as follows. Wool fibers were suspended in 0.2 M sodium thioglycolate, pH 12, at a 50:1 vol/wt ratio in a stoppered flask. The mixture was stirred overnight at ambient temperature, 21˚C - 23˚C. On the next day, the mixture was diluted with an equal volume of deionized water, and the pH was reduced to 10.5 with 0.5 M HCl, and the mixture was centrifuged at 15,000 g for 30 min. The supernatant was adjusted to pH 7 with dilute HCl and oxidized with 0.25 ml 3% H_2O_2 per ml supernatant for 3 h. The pH was then adjusted to 4 with 0.5 M HCl and a solid fraction was allowed to precipitate overnight.

2.4.4. Denaturation/Reduction—Urea/2-Mercaptoethanol (UTM)

The Shindai method developed by Nakamura *et al.* [11] for extraction of keratin from human hair was adapted as follows. Briefly, wool fibers (0.5 g) were incubated for 3 days at 50˚C and 100 rpm at pH 8.5 in 125 ml of 25 mM Tris, 2.6 M thiourea, 5 M urea, and 5% 2-mercaptoethanol. At the end of 3 days, the mixture still had a large fluffy component that was centrifuged out at 15,000 g for 30 min.

2.4.5. Denaturation/Reduction—Urea/ Metabisulfite (SMB)

The method of Isarankura Na Ayutthaya *et al.* [16] for extracting keratin from chicken feather waste was adapted. Briefly, wool fibers (0.5 g) were incubated for 5 h at 65˚C, 120 rpm in 35 ml of a solution containing 8 M urea, 0.25% SDS, and 2 g sodium metabisulfite.

2.4.6. Reduction—Sulfide (SUL)

The method of Feairheller *et al.* [17] for solubilization of cattle hair was adapted. Briefly, wool fibers (1 g) were soaked in 150 ml deionized water for 20 min, then 1 g sodium sulfide was added for 5 min, followed by 3.2 g calcium oxide (lime). The mixture was gently stirred for 48 h.

2.5. Electrophoresis

Lyophilized samples (~1.0 mg) were dissolved in electrophoresis buffer (1 mM EDTA, 2.5% SDS, 5% 2-mercaptoethanol and 0.01% bromophenol blue) for analysis by SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulfate). The samples were heated at 90˚C for 15 min. Separation was achieved on a PhastGel System (GE Life Sciences, Piscataway, NJ) using the standard protocol for protein on 20% homogenous gels. A broad range SDS-Standard (Bio-Rad, Hercules, CA) containing nine proteins ranging in size from 6500 to 200,000 Daltons was included as a control on each gel. Gels were stained with Coomassie Brilliant Blue R.

2.6. Economic Analysis

The solubilization methods were compared with respect to efficiency in terms of the number of operator steps and the overall length of time required. For a relative cost estimation, the prices per gram of wool, of chemicals other than dilute acids or bases were compared, and any requirement for additional heat was noted. Additional considera-

tions were operator safety, and the probable ease of scaling the process up.

3. Results and Discussion

Wool that has been cleaned (scoured) for commercial processing into yarns and textiles, retains some residual lipid that must be removed prior to solubilizing the keratin [18]. There are nearly as many protocols for removal of residual lipid as there are researchers on the topic of soluble keratin. The aim of this research was to develop a reasonably benign, safe and cost effective procedure that could effectively clean the wool of any residual lipid. Intuitively, a surfactant wash such as that used for human hair [10] and wool [19] seemed like a good choice. However, when these wool fibers were treated with 0.1% TWEEN20, the result was a wool fiber clump that could not be washed free of surfactant. The chloroform/methanol treatment for human hair [11] was ineffective for wool fibers, which tended to form clumps that required milling prior to further processing. Soxhlet extraction of 1/4 in. fibers with petroleum ether [12] for 6 h removed ~3 mg lipid per g wool and produced a sample easily used for further experiments. Soxhlet extraction with hexane, a solvent frequently used for lipid extractions that is somewhat less flammable than ether, proved equally effective, removing slightly more than 3 mg lipid per g wool.

Results of triplicate analysis of this wool fiber sample before and after hexane extraction [9] are shown in **Table 1**. Crude protein 90.79% ± 0.38% was determined on the hexane extracted wool and calculated from total nitrogen using a factor of 6.06, estimated from the amino acid analyses of Freddi *et al.* [20] and Jones [21].

3.1. Solubilization

With the exception of the peracetic acid oxidation and the urea/mercaptoethanol denaturation/reduction methods, wool fibers disappeared from the reaction vessel and were not retained on paper during the initial filtration. Both supernatant and solid fractions resulting from centrifugation were comprised of material that was extracted from the fibers with varying degrees of solubility under the final conditions. A comparison of the solubilization methods in terms of the weight of solubilized keratin per gram of wool fiber was hindered by the small size of the samples and electrostatic forces that caused protein to adhere to the lyophilizing flasks.

3.2. Electrophoresis

Electrophoresis was carried out on 20% homogeneous gels in anticipation that most of

Table 1. Analysis of wool.

Sample	Moisture %	Ash[a] %	Lipid[a] %
Raw wool	7.06 ± 0.70	0.82 ± 0.30	1.20 ± 0.51[b]
Hexane extracted	8.68 ± 0.21	1.48 ± 0.17	2.93 ± 0.03[c]

[a]On a dry weight basis; [b]Determination for raw wool was by HCl hydrolysis followed by $CHCl_3$ extraction; [c]Determined from the weight of lipid removed from wool by Soxhlet extraction with hexane.

the soluble keratin would be hydrolyzed to the 5 to 25 K molecular weight range [22]. The methods employed in this work were milder, and produced a wider molecular weight range of keratin fragments. Not surprisingly, given the nonhomogeneous nature of wool protein, no single molecular weight fragment was observed. Peracetic acid (PAA) oxidation produced the largest soluble fragments with molecular weights >60 K, and no low molecular weight fragments that were retained on the gel (**Figure 1**, lanes 2 and 3). Percarbonate (PCC) oxidation produced fragments in the 50 - 200 K range (**Figure 1**, lanes 4 and 5). The TGA method that reduces the keratin with thioglycolic acid and then uses hydrogen peroxide to oxidize the solubilized protein produced fragments (**Figure 1**, lanes 6 and 7) across the entire 6 K to 200 K range, with most material in the 40 - 70 K range characteristic of intermediate filaments [23].

Two methods that rely primarily on protein denaturation gave distinctly different results. The SMB procedure, urea denaturation with reduction by sodium metabisulfite, produced mostly fragments with molecular weights less than 30 K (**Figure 2**, lanes 2 and 3). In contrast, urea/thiourea denaturation with 2-mercaptoethanol reduction (UTM) produced the sharpest bands in the 60 K - 80 K MW range (**Figure 2**, lanes 4 and 5) as predicted by Deb-Choudhury *et al.* [8], and also a smear of small fragments in the 6 K to 14 K range. Reduction with sulfide (SUL) produced fragments across the detectable molecular weight range with distinct fractions, in the 66 K, 40 - 50 K, and <20 K ranges (**Figure 2**, lanes 6 and 7). In all cases, the similarity in the gel patterns between supernatant and solid fractions from centrifugation suggests that fractions that are extracted under solubilizing conditions may not remain in solution when the conditions are changed.

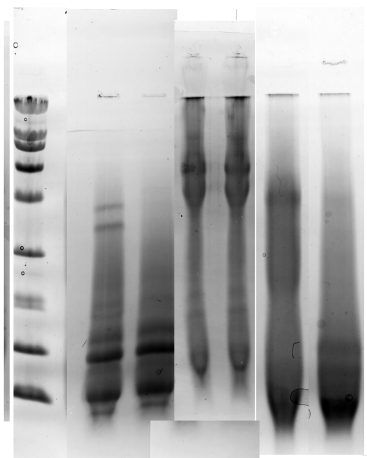

Figure 1. Lanes 1 and 8 represent the broad range standard containing 9 proteins with molecular weights at 200.0 K, 116.2 K, 97.4 K, 66.2 K, 45.0 K, 31.0 K, 21.5 K, 14.4 K, and 6.5 K. Lanes 2 and 3 are solution and solid fractions of peracetic acid oxidized keratin. Lanes 4 and 5 are solution and solid fractions of percarbonate-oxidized keratin. Lane 6 is thioglycolate-reduced keratin, and lane 7 is thioglycolate/hydrogen peroxide reduced/oxidized keratin. The picture is a composite of separate SDS- PAGE experiments on 20% homogeneous gels for each treatment.

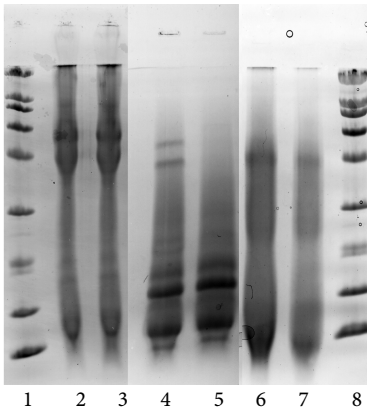

Figure 2. Lanes 1 and 8 represent the broad range standard containing 9 proteins with molecular weights from 200.0 K to 6.5 K. Lanes 2 and 3 are solution and solid fractions of urea/thiourea denatured mercaptoethanol-reduced keratin. Lanes 4 and 5 are solution and solid fractions of urea/sodium metabisulfite reduced keratin. Lanes 6 and 7 are solution and solid fractions of sulfide-reduced keratin. The picture is a composite of separate SDS-PAGE experiments on 20% homogeneous gels for each treatment.

3.3. Economic Comparison

Each of these methods is viable, in terms of cost of materials, energy input, and time at the research scale. When planning to scale the method up to the pilot plant or industrial level, differences become more important. The relative costs are compared in **Table 2**. In making this comparison, we have ignored the dialysis and lyophilization steps that are common to all of the methods and that could well be replaced by other ways of preparing for utilization of the soluble keratin. The comparison of costs of chemicals is based on the price from a single supplier of reagent grade chemicals for extraction of soluble protein from 1 g of wool. The methods are ranked 1 - 6 on the assumption that when the process is scaled up, and the chemicals are technical grade, the relative prices will still be similar. Actual prices are not shown because they will vary over time and will depend on supplier availability. Overall, the input costs can be grouped in three sets. The percarbonate oxidation and sulfide reduction methods, which were originally developed for removing hair from cattle hide in preparation for leather tanning, have the lowest costs in terms of chemical and energy input. Thioglycolate reduction followed by hydrogen peroxide oxidation, and peracetic oxidation, both methods developed for solubilizing keratin, have intermediate costs with the major factors being storage of thioglycolate at −80˚C and the higher temperature required for the peracetic acid reaction. The highest input costs are for the urea/metabisulfite and urea/thiourea/mercaptoethanol methods where chemical prices are an order of magnitude higher than for the other methods evaluated, and the requirement for an elevated temperature is also greater.

At the bench scale, each of these methods can be safely and effectively carried out. In judging whether or not it is practical to scale a process up to the pilot plant or industrial scale, factors in addition to the costs of chemicals and energy may become important.

Table 2. Economic factors.

Method	Price[a]	Days	Steps	Temp[b]	Scale[c]
Oxidation/PCC	1	1	3	RT	++
Reduction/Sulfide	2	2	3	RT	++
Reduction-oxidation/TGA	3	3	5	RT	+
Oxidation/PAA	4	3	5	37	+
Denaturation-reduction/MBS	5	1	1	65	−
Denaturation-reduction/UTM	6	3	3	50	−−

[a]Relative cost of chemicals on a 1 g of wool basis; [b]Required temperature in ˚C for the reaction; [c]Feasibility of scaling up the method, based on cost of reagents and energy input, as well as level of training needed for safe operation of the process, ++ means most feasible, −−means least feasible.

For example, working with small volumes of hydrogen peroxide for bench scale oxidation reactions is safe, but on a larger scale, the solid percarbonate, a typical component of nonchlorine laundry bleaches, would be a safer option for oxidation. In addition to safety, the ease of working with the reagents is a consideration. In this study, the ease of performing the procedure happened to decrease in parallel with increasing costs.

4. Conclusion

Six published methods for extracting keratin from meat animal byproducts and human hair were adapted for use with low quality wool fibers. Varying molecular weight fractions of keratin were obtained with the different methods, the urea/metabisulfite method produced mostly small fragments 6 K - 10 K MW, while the oxidative methods produced fragments with molecular weights greater than 60 K. Economic considerations such as the necessity of elevated thermal energy and the cost of materials were considered. Percarbonate oxidation and sulfide reduction, originally developed for removal of hair from cattle hide, performed comparably to methods intended for wool, human hair, or feathers. The data presented is expected to aid the scientist in deciding the method or the further adaption of which method is likely to produce the optimal component for the envisioned product.

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