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Review Article

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Recent Developments in Alternative Extraction Processes of Keratin from Keratinous Animal Body Parts as Active Ingredient for Hair care Products

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Abstract

Recently, keratin has been attracting intense attentions as an active ingredient for hair care products, given its unique nature such as rejuvenating and anti-aging characteristics. Conventionally, keratin is extracted from keratinous animal body parts (KABPs) such as wool by chemical processes or in combination with enzymes. However, for extraction of keratin from KABPs requires large volumes of highly concentrated toxic chemicals, which is not environmentally sustainable. The time-consuming processes for the extraction and the removal of chemicals slow down the productivity. Lately, a number of alternative extraction processes have been reported. We will review these processes and examine their commercial viability. We also report the recent development in our alternative process based on thermal hydrolysis.

Keywords: Keratin • Thermal hydrolysis • Molecular weight distribution • Cystine • Hair care

Introduction

Keratin has been increasingly used as an active ingredient for haircare and skincare products. It is also known to have rejuvenating and anti-aging effects on hair and skin. In fact, keratin accounts for more than 60 percent of all protein-containing haircare products, according to Global New Products Database. What distinguishes keratin from any other protein is its significantly high degree of the sulfur-containing cystine which is higher than any other protein. For example, keratins typically contain 4~12 cystine residues per 100 amino acid (AA) residues, whereas collagen typically contains less than 1 residue per 100 residues. Cystine is an important AA residue to maintain a protein structure, providing disulfide bonds as crosslinks inside a protein or between two protein molecules. Cysteine (Cys-SH), the reduced cystine residue, is a component of a well-known antioxidant, glutathione. Currently, however, a majority of keratin ingredients are extracted by chemical processes which use large volumes of highly concentrated, sometimes toxic chemicals, raising environmental concerns. In this review, we discuss the recent developments in extraction of keratin. In particular, we focus on thermal hydrolysis process (THP) as an alternative extraction process which seems a promising extraction process, given its potential to leave much less environmental footprints and increased productivity, compared to the conventional chemical processes. First, we begin with the keratin's unique characteristics as ingredients for cosmeceuticals.

Keratin as Cosmeceutical Ingredient

Keratin is an intracellular protein in animal-body parts such as hairs, wool, nails, skins, feathers, hooves, claws, and others. They are called keratinous animal body parts (KABPs) and usually generated at rendering plants where animal body wastes are brought in from slaughterhouses and recycle them as much as they can as animal feeds and other by-products. However, KABPs are very difficult to recycle, given their recalcitrance for valorization. As a result, mostly they are disposed of at landfills where they are eventually decomposed overtime, generating methane emissions. Recently, valorization of these animal body wastes is receiving intense research [1-4]. With the growing global demand for meats, generation of KABPs will only increase, ensuring a stable supply of KABPs as raw materials to whatever they are turned into. Currently, cosmetics and biomedical applications of keratin are attracting strong attentions from researchers and industries among others.

Figure 1 illustrates the cascading structure of hair shaft which consists of cuticle, medulla, and cortex which is made of many macro fibrils each of which is composed of micro fibrils. Though this is a well-known structure, it is relevant to the extraction of keratin. Inside micro fibrils, there are a number of intermediate filaments (IFs), each IF consisting of several tetramers, each of which are composed of four keratin α-helices, two of them pair up one another, forming the coiled coil structure, independently discovered by Linus Pauling [6] and Francis Crick [7], a basic structural element of keratin fibrils.

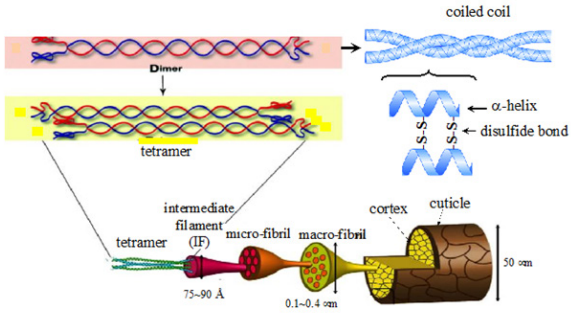

Figure 1. The hair shaft structure consisting of cuticle, cortex, and medulla which is located at the inner most layer, thus not shown. Dimer is the coiled coil made of two a-keratin helices twisted around one another. Two a-keratin helices are crosslinked by the disulfide (-S-S-) bonds of cystine. Dimer packs itself with another dimer, forming tetramer which makes up the intermediate filament (IF). A part of the figure is taken from ref 5.

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The coiled coil is formed through the disulfide (-S-S-) bonds of cystine between the two α-helices. The hydrophobic groups of the constituent AAs of each α-helix are wrapped around inside the coiled coil structure, contacting one another between the two α-helices, strengthening the coiled coil structure. The hydrophobic groups on the outer surface of the coiled coil also allow very tight packing of two coiled coils through the hydrophobic interactions, forming the tetramers which are packed into the micro fibrils which are crammed into the macro fibrils [8]. Hence, what makes keratin recalcitrant to extraction are 1) the disulfide bonds covalently connecting two α-helices, forming the coiled coil and 2) the hydrophobic interactions stabilizing the coiled coils and packing them into IFs. Conversely, the two key forces, the disulfide bonds and the hydrophobic interactions, need to be removed or lessen in order to extract keratin from KABPs. Breaking hydrogen bonds forming α-helix is an additional means to unlock the tightly packed IFs since the removal of hydrogen bonds can denature the keratin protein. The disulfide bond, though it is stronger than a hydrogen bond, having the bond dissociation energy of 60 kcal mol^{-1}, is not as strong as the C−C and C−H bonds having 40 % more dissociation energy than the disulfide bond. Hence, the S−S bond is susceptible to scission by polar reagents, both electrophiles and especially nucleophiles (Nu):

$$RS{-}SR + Nu^{-} \rightarrow RS{-}Nu + RS^{-} \quad (1)$$

On the other hand, though the hydrophobic interaction between two AA residues is not very strong, since a protein has a long chain, the collective hydrophobic force among many AA residues along the protein chain can mount to a large force. This is one of the foundations for stabilizing the coiled coil structure.

Once extracted, keratin ingredients for cosmetic applications are required to have a wide range of MW distribution. For example, a patent by Keraplast Technologies LLC (KTL) describes a process to extract keratin of high MW (10 KDa~60 KDa) from wool and the extracted keratin is used for cosmetic applications [9-10]. Likewise, Cardamone et al. describe a process of extracting keratin with even a wider MW distribution, 100 Da ~ 100 KDa, for cosmetic application [11]. Another patent application US5679329A claims keratin ingredients with MW up to 200 KDa as an ingredient for hair care products [12]. The rational for having a wide range of MW distribution for keratin used in haircare products is that the low MW fractions can penetrate through the cortex to restore the keratin fibrils, while the high MW fractions can cover the surface of hair as coating. Below, we first review the current chemical processes, then discuss THP as the alternative extraction process and compare the benefits of THP against the chemical processes.

Chemical Process

We have chosen two chemical processes: the Shindai method and the processes developed by KTL, a leading keratin manufacturer. The former is known to be one of the most common extraction protocols [13]. It gives one of the highest keratin recovery yields, ~ 75%, from a variety of KABPs [14]. The recovery yield, Y, is defined as follows:

$$Y=[W_{Keratin}]/[W_{KABP}] \quad (2)$$

where $W_{Keratin}$ and W_{KABP} are the weights of the extracted keratin and the original keratin in KABP, respectively. The protocol involves incubation of the samples at 50°C for 2 days in a buffer consists of 20 mM Tris-HCl (pH 8.5), 2.6 M thiourea, 5 M urea, and 5% (v/v) 2-mercaptoethanol (2-ME). Urea and thiourea work as the chaotropic agent to swell the fibril network by denaturing the hydrophobic interactions between α-helices, unwinding the coiled coil structure and also by breaking intra- and intermolecular hydrogen bonds of α-helices. Interfering with hydrophobic interactions of protein by urea has been well-documented [15,16]. 2-mercaptoethanol, on the other hand, is used as the reducing agent to break the disulfide bonds, according to eq 1. In fact, it is frequently used to reduce the disulfide bonds of protein to denature. The extracted keratin by the Shindai method had 9.3 mole % of cysteine residue and the MW distribution of 12 KDa ~ 135 KDa [17]. Both the recovery yield and the cysteine residue content are high, compared to other chemical processes, suitable for cosmetic applications. However, the high concentrations of urea and thiourea raise health as well as environmental concerns. Though the concentration is not as high, 2-mercaptoethanol belongs to Category 1 in four areas: liver and heart acute aquatic toxicity, chronic aquatic toxicity, serious eye damage, and skin sensitization, according to GHS hazard classification [18]. The time-consuming process of the chemical methods in general is another issue. The Shindai method requires 2 days for extraction of keratin from KABP, followed by repeated dialysis against distilled water for filtration [14]. According to the recent economic analysis of keratin hydrolysis methods by USDA-ARS, an effective keratin hydrolysis by a combination of chaotropic agent and reducing agent is not promising for economic scale up productions primarily due to the high cost of chemicals [19].

On the other hand, KTL uses oxidative sulfitolysis for extraction of keratin from wool [9]. One process uses 2 kg of copper sulfate pentahydrate, 8 L of concentrated ammonia, and 5 kg of sodium sulfite, each of which is a Category 2~3 toxic chemical, a Category 1 toxic chemical, and a Category 2 ~ 2A toxic chemical, respectively, for every 10 kg of wool from keratin is extracted. In addition, the process requires 24 hrs of agitation for extraction and the total of 120 hrs of filtration [9] The extracted keratin has 4.2~12.4 mole % of S-sulfonated cysteine (Cys-S-SO_3H), high cysteine residue contents. The S-sulfonated cysteine, as is the case for cysteine, is capable of cross-linking with other cysteine residues through disulfide bonds. It is assumed this cross-linking between cysteine residues repairs damaged hair. However, the cross-linking must occur at right residue locations along the keratin protein chain. A risk is that the cross-linking may be formed at wrong locations, given the tendency of the S-sulfonate group or the thiol group to react with the nearest cysteine or the S-sulfonate group, regardless of the AA residue locations. This can result in a misfolded keratin protein or coiled coil. Once the disulfide bonds are cleaved by sulfitolysis, the S-sulfonated cysteine has no memory of the native location where the disulfide bond was formed before the cleavage. In fact, it has been reported that the misfolding or aggregation of protein due to the reshuffling of the disulfide bonds after unfolding of protein through reduction of disulfide bonds [20] (Figure 2).

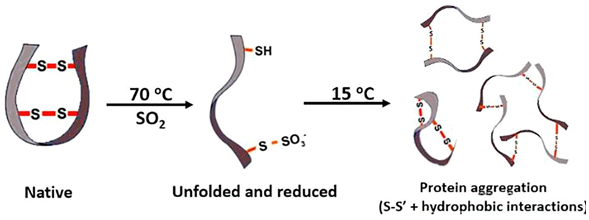

Figure 2. Proposed mechanism for protein misfolding and aggregation through aberrant scrambled formation of the disulfide bonds after unfolding of protein by disulfide bond cleavages. Model proposed for protein misfolding and aggregation through aberrant scrambled formation of the disulfide bonds. The intermolecular disulfide bonds before unfolding became both intermolecular and intramolecular disulfide bonds after unfolding. The figure is taken from ref. 20.

No recovery yield is available for the KTL process. FK Restore™ is one of the haircare products manufactured using the keratin extracts from wool by the KTL process. It had the MW distribution of a few hundred Da to >100 KDa [21]. The price is high, $1,785/kg of keratin, reflecting the costly extraction and purification processes. It is worth mentioning that KTL also promotes another keratin called Oxidized Keratin extracted from wool by an oxidation process. During the oxidation process, the disulfide bond of cystine is broken up, leaving cysteine with the SO_3H group, or cysteic acid. Cysteic acid is not capable of cross-linking with other cysteine residues unlike the S-sulfonated cysteine. Yet, KTL has shown that Oxidized Keratin is very effective in repairing damaged hair [10]. Their article seems to contradict with the claimed functionality of the S-sulfonated cysteine in repairing damaged hair by KTL. Further study is warranted to investigate the effectiveness of the cysteine residue capable of crosslinking on haircare treatments.

Before we discuss THP, we briefly review other alternative extraction processes reported in the literature. Ionic liquids have been successfully applied to extract keratin protein with a high extraction yield due to their ability to solvate keratin fibrils [22]. Yet, the high recovery cost of ionic liquids for reuse and their toxicity hinder a wide application of this method. Enzymatic hydrolysis is also time-consuming and high in cost. Microwave has also been used to extract keratin from wool [23]. The extraction yield varied from 5% to 60%, depending on the temperaure of water in an autoclave inside the microwave and the weight ratio of KABP to water inside the autoclave. The content of either cysteine or cystine in the extracted keratin ranged from 0.1 to 1.6, and the MW distribution was between 3K and 8 KDa.There was no high MW fraction in the extracted keratin observed by SDS-PAGE. In addition, scaling up of microwave process may be problematic. Steam explosion has been applied to extraction of keratin from wool at 220 ºC for 10 min [24]. However, the resulting products showed disruption of the histological structure and reduction of the molecular weight (MW), giving rise to small peptides and free AAs. No recovery yield was reported.

Thermal hydrolysis as alternative extraction process

Water has very unique characteristics in that it is a very good solvent for electrolytes or hydrophilic molecules, while it is a poor solvent for hydrophobic molecules such as fats or hydrocarbons at ambient temperature. However, the reverse is true at high temperatures. It becomes a good solvent for aromatic hydrocarbons [25]. This is due to a significant reduction in the dielectric constant of water at high temperatures: it drops by almost half in going from 20°C (α=78.5) to 150°C (α=43.2) [25]. That is, water becomes less polar as the temperature increases. It follows that water favors hydrophobic interactions at high temperatures. In other words, water interferes with the hydrophobic forces tightly packing keratin fibrils, loosening the fibrils and swelling them at high temperatures. This process unlocks one of the keys forces, the hydrophobic interactions between α-helices, coiled coils and eventually fibrils. With the polarity becoming less at high temperatures, hydrogen bonds may be weakened, denaturing the keratin α-helices. This process may be equivalent to what a chaotropic agent does to keratin fibrils in a chemical process. The changes in the dynamic viscosity, the surface tension, and the self-diffusion constant of water at high temperatures also facilitate the wetting and the mass transfer of the components in keratin fibrils [25]. We refer to this temperature as T1 in our THP method.

The next step is to unlock another key defense to keep the keratin fibril intact, cleaving the disulfide bonds. Water has another unique characteristic at high temperatures: its dissociation constant, $K_w=[H_3O^+][OH^-]/[H_2O]^2$, increases by almost two orders of magnitude in going from 20°C to 150°C, for example [25]. This brings down pH of water to 5.7 at 150°C, making water acidic, and increasing the concentration of H_3O^+. With the keratin fibril structure swollen and the α-helices denatured, pores or channels may be created in the macro or microfibrils for H_3O^+ as the oxidizing agent to diffuse through the fibril network eventually to reach the disulfide bonds for cleavage. We refer to this temperature as T_2. By adjusting the reaction temperatures, T_1 and T_2, which change the reaction kinetics, and the reaction time, a degree of oxidation of the disulfide bond can be controlled. This is our two-step heating process which gives us more flexibility in adjusting the keratin extraction process and hence, controlling the keratin characteristics such as the cystine content, the MW distribution, and the recovery yield, than a one-step heating process. By the two-step heating process, we may be able to either increase the recovery yield through rigorous extraction or to preserve the original characteristics of keratin under moderate conditions, using the 1st heating step as the pretreatment.

There are three previous articles on keratin extraction using THP in the literature [26-28]. Yin et al. have used THP at 220°C for 2 hrs to extract keratin from feather barbs and reported the MWs of the hydrolysates were only about 1 K~1.8 KDa [26]. Bhavsar et al. have reported the extraction of keratin from wool by THP in a combination with 1~5% of alkali agent at 140°C or 170°C for 1 hr. [27]. They extracted keratin hydrolysates with 3 K~8 KDa of MW with or without the alkali. Esteban et al. have studied hydrolysis of hog hairs in order to breakdown to AA by THP [28]. The temperature range for the hydrolysis was 200~300°C. They found that the AA production from hog hairs reached the maximum of 35% with respect to the protein in the original sample at 250°C. No other hydrolysates were reported; hence no MW distribution is available.

What is common among all three previous studies on THP is that the MW of the extracted keratin was only up to 8 KDa which may be too low for haircare applications, given high MW-keratin ingredients used in haircare products already in the market. The cystine residue/cysteine residue contents were either none or nearly zero, depending on the condition. This is the result of oxidative decomposition of cystine at high temperatures used in these studies. Moreover, none of the study reports the recovery yield. They all used a one-step heating process.

Keratin Extraction

Our keratin extraction and recovery system consists of two processes: THP and the shear wave-induced ultrafiltration (UF). After THP even at very high temperature, there are still small pieces of leftover hair, <1 μm, that need to be removed from the reaction solution. Hence, UF is required to remove them. Since UF with a 150 KDa membrane removes all suspended solids, the permeate from UF only includes dissolved solids, solubilized keratin hydrolysates in this case. The shear wave-induced filtration generates a vortex flow inside the membrane chamber to prevent membrane fouling. Since protein tends to cause fouling, this filtration system is ideal. The detail of the procedure and the analytical instrumentation have described elsewhere [21].

Most of the keratin ingredients in keratin-based haircare products in the market are extracted from wool. However, any KABPs can be used as the source in principle. We used hog hair as the sample for our process. Figure 3 compares the AA compositions for human hair, wool, and hog hair in mole % on dry matter basis. Those for human hair and hog hair were determined by using the conventional AA analysis using HPLC. The composition for wool has been taken from a literature [29]. The AA composition is an important measure in comparing the AA sequence which determines the keratin protein structure. The composition for tryptophan is not shown due to its decomposition during hydrolysis prior to the HLPC analysis of AAs. Hence, it is a procedural artifact. Some differences are observed among the three samples: glycine and leucine contents of wool are considerably higher than those of human hair.

The standard deviations of the AA composition of wool and that of hog hair from that of human hair are 1.6 and 1.0 mole%, respectively. Hence, the AA composition of hog hair is somewhat closer to that of human hair. The THP conditions can be adjusted to meet the target required for a given application. Here, we focused on two targets: a wide MW distribution and preservation of the cystine residue in the extracted keratin. Accordingly, we chose the following THP condition: T1=100 °C for 3 hrs. and T2=140°C for 2 hrs. The extracted keratin under this condition will be referred to as KH in this work.

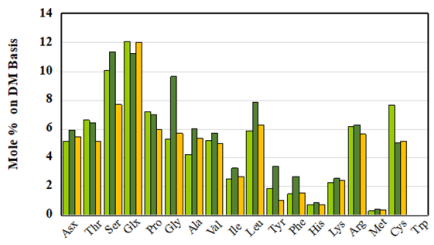

Figure 3. Comparison of the AA compositions for human hair (the light green bars), wool (the dark green bars), and hog hair (the orange bars) in mole % on dry matter basis. Asx refers to either aspartic or asparagine or both, and Glx represents either glutamic acid or glutamine or both.

MW Distribution

Figures 4 and 5 display SDS-PAGE charts and MALDI-TOF mass spectroscopy charts for the keratin ingredient in FK Restore™ and KH. According to SDS-PAGE, FK Restore™ shows high concentrations of MW distribution over 40 KDa, below which the bands start fading. On the other hand, KH exhibits very thick, continuous bands from the top to the bottom of the chart. As to the low MW region <1 KDa, KH shows more peaks for AAs and oligopeptides than the keratin ingredient in FK Restore™ does (Figure 5). Together, both Figures 4 and 5 demonstrate that KH has a very wide MW distribution from a few hundred to >100 KDa.

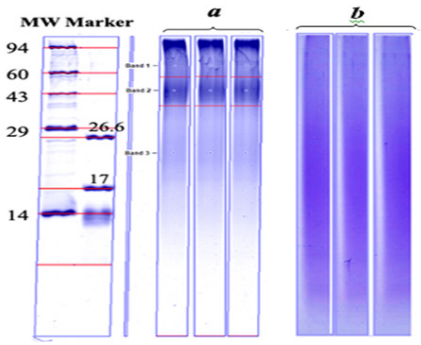

Figure 4. SDS-PAGE charts for (a) the keratin ingredient in FK RestoreTM from KTL and (b) KH. The numbers shown below MW Maker are the MW for the corresponding band.

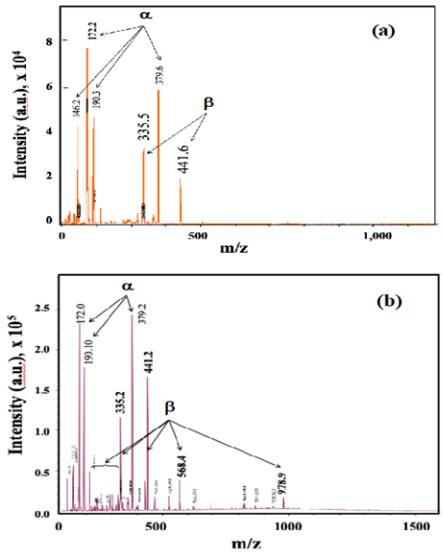

Figure 5. MALDI-TOF mass spectra for (a) the keratin ingredient in FK Restore™ and (b) KH. α: the peaks due to a-cyano-4-hydroxycinnamic acid which is the matrix used for MALDI-TOF mass spectroscopy and β: the peaks due to the samples.

AA Composition

Figure 6 compares the AA compositions of human hair, the keratin ingredient in FK Restore™ and KH. The standard deviations for the AA composition of the keratin ingredient in FK Restore™ and of KH from that of human hair are 2.4 % and 1.5 %, respectively. Hence, the AA composition of KH is closer to that of human hair. The results clearly demonstrate that KH preserved some cystine residues, 2.6 mole % which is about half the cystine residue in the original hog hair, 5.2% (Figure 3).

Despite our intense literature search, we failed to find any study showing any cystine residue left in extracted keratin by chemical process or otherwise. The keratin ingredient in FK Restore™, on the other hand, show no cystine content. It does have high cysteine content in the form of S-sulfonated cysteine, while KH has no cysteine content due to the nature of THP which oxidizes cysteine to cysteic acid. Despite the claimed benefit of the S-sulfonated cysteine capable of forming cross-links with other cysteines, the risk of cross-linking at wrong AA residue locations has been pointed out earlier, resulting in misfolding of keratin protein. On the other hand, the cystine residue in KH likely preserves the original tertiary structure of keratin, avoiding the risk. In addition, some disulfide bonds maintained through cystine in KH provide the structural integrity which may help restore damaged hair.

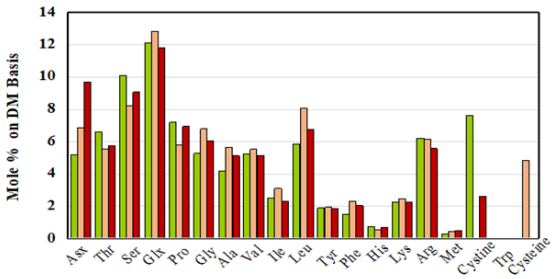

Figure 6. Comparison of the AA compositions for human hair (the light green bars), the keratin ingredient in FK Restore™ (the orange bars), and KH (the red bars) in mole % on dry matter basis. Asx refers to either aspartic or asparagine or both, and Glx represents either glutamic acid or glutamine or both.

We speculate that the preservation of the cystine residue was made possible by our two-step heating THP process. The THP condition used in this work may have provided an environment where hair was slowly heated at relatively low temperature long enough to swell the keratin fibrils thoroughly, followed by heating at a moderate temperature, not too high to oxidize all the disulfide bonds, but low enough to preserve some disulfide bonds, while breaking just enough disulfide bonds to separate the keratin protein from the fibril network to solubilize it. As eq 1 indicates, for the cleavage of the disulfide bond, nucleophiles are often required. Sulfites are well-known nucleophiles often used to break up the disulfide bonds. In water, on the other hand, there are more electrophiles, H_3O^+, than the nucleophiles, OH^-, at high temperatures. THP is a hydrolysis process; hence, it is an excellent tool for hydrolyzing protein to peptides. The THP condition may have been just enough to hydrolyze keratin protein to peptides, releasing them from the fibrils, while maintaining some cystine residues. Figure 7 illustrates a schematic model of a possible KH without any structural analysis. This pair could form a part of a damaged coiled coil. Still no structural instrumentation work has been performed to prove this structure, hence, purely speculative.

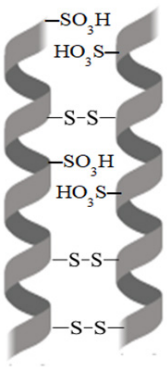

Figure 7. A schematic model of a possible KH structure. No structural instrumentation work was performed to prove this structure.

On the other hand, the recovery yield of KH was modest, ~45%; however, the THP condition was not optimized yet. A study has reported that the recovery yields of keratin from wool by some chemical processes were 53, 41, 25, and 5 % for the reduction, the sulfitolysis, the alkali, and the oxidation methods, respectively [30].

From the point of view of the profitability of the operation, a low recovery yield, i.e., a lower production volume than expected, may not

matter as much when the raw material, hog hair in this case, is virtually free, as compared to sourcing raw materials from a supplier. Besides, the leftover hair still contains plenty of protein and can be reprocessed by THP at higher temperatures to extract more keratin for other products such as food additives or possibly another hair care product as well. The keratin hydrolysate extracted by THP at high temperatures >180°C loses both cystine and cysteine residues [21]. Still, Oxidized Keratin by KTL not capable of crosslinking is marketed as an ingredient for haircare products. Our previous study showed the recovery yield of 70% at T_1=100°C for 1 hr. and T_2=200°C for 1 hr [21].

On the productivity, it takes only one day to process 10 kg of hog hair including separation and purification, using our process. The extraction takes only 2 hours, compare to 2 days [14] or 24 hrs [9]. The purification requires about 2 hours, as opposed to 120 hrs [9]. With an about 50% of recovery yield, the productivity would be 5 kg/day. On the other hand, the KTL process requires 144 hrs for extraction and purification of keratin from 10 kg of wool. The productivity would be 1.7 kg/day, assuming 100 % recovery yield. The recovery yield of the KTL process is not known.

Conclusion

We have reviewed alternative processes to extract and recover keratin from KABPs. Among them, THP is a promising process, leaving less environmental footprints than chemical processes. The operation cost can be less as well, using water as the major extraction solvent. Yet, so far, no commercialization of THP for keratin extraction has been realized. The previous THP studies showed only low MW distributions which may not be ideal for haircare applications. In this review, however, we have demonstrated that our two-step heating process can yield keratin extracts not only with a wide range of MW distribution, but a significant content of cystine residues preserved from the original KABP. Keratin extracted from KABP has yet to be reported to have the cystine residue left by chemical process or otherwise. Before commercialization of our process, however, the efficacy test of KH on human hair need to be conducted.

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