

The Use of Derived Keratin-Associated Proteins as a Potential Treatment for Damaged
Hair through Disulfide Bond Reformation

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Abstract

This study investigated the effect of keratin-associated proteins on the strength of previously damaged hair. During the formation of a hair fiber, keratin-associated proteins (KAPs) disulfide bond with keratin intermediate filaments alongside other KAPs. These disulfide bonds are a crucial factor in the structure of hair fiber and are needed to maintain strength. Past research has identified that in specific hair treatments, such as bleaching, or in genetic hair diseases, these disulfide bonds are either broken or not present, hence weakening the hair. In this research, through chemical treatments, KAPs were able to be extracted from healthy hair fibers and made into a solubilized solution named ReKAP. This solution was applied to untreated hair fibers and damaged hair fibers, bleached and heated, to determine the effects on strength. The strength of the hair was analyzed through tensile testing, normalized, and compared against other groups. In this study, it was observed that ReKAP had no statistically significant effect on the ultimate tensile strength and strain of untreated and heated hair; however, results indicated that ReKAP had a statistically significant effect on increasing the strength of bleached hair. This suggests that in the damaged hair cortex due to the bleaching process, small-molecule KAPs have the ability to fill up, seal, and crosslink to restore the damaged hair structure. Thus, ReKAP and other forms of solubilized KAPs have the potential to revitalize the strength of damaged hair or possibly prevent damage from occurring in the future.

Introduction

The objective of this research study was to investigate the use of keratin-associated proteins (KAPs) derived from hair fibers as a treatment to reinforce previously-damaged hair strands. Human hair keratin is known to be one of the main structural components of hair alongside its associated proteins, otherwise known as KAPs. It makes sense to repair damaged hair with one of its vital structures.

There are three classes of KAPs: high-sulfur proteins (HS), ultra-high-sulfur-rich proteins (UHS), and glycine and tyrosine-rich proteins (HGT) (Fraser & Parry, 2018). In previous studies, KAPs have been shown as essential components in the formation of hair fibers which is complex involving several processes and different structures, hence contributing to the overall strength of the complete hair fiber (Fraser & Parry, 2018).

The structure of hair is composed of three main portions: an outer cuticle cell layer, an inner cortex, and a central medulla (Barthélemy et al., 2012). The majority of the hair's mass is located in the cortex, which is composed of different types of cells that each contain around 500-800 keratin intermediate filaments, otherwise known as KIFs (Barthélemy et al., 2012), as well as multiple KAP molecules. KIFs undergo an internal reorganization that enables intra-KIF disulfide-bond formation (Harland et al., 2022). During this process, disulfide bonds between KIFs and KAPs form alongside disulfide bonds between KAPs and KAPs (Harland et al., 2022). The disulfide bonds formed during this process are known to have a significant contribution to the strength of the hair fiber (Fraser & Parry, 2018). Moreover, in previous studies, it has been identified that disulfide bonds break under tensile stress (Harland et al., 2022). Therefore, KAPs have a pivotal role in the formation of disulfide bonding, which has a significant contribution to overall hair strength.

There are several hair diseases that may be linked to a decrease in the amount of KAPs present in the structure of the hair. One of these diseases is trichothiodystrophy, which is often characterized by photosensitivity, ichthyosis, and brittle hair (Rogers et al., 2006). Previously, there have been several gel electrophoresis studies concerning trichothiodystrophy which have illustrated that patients with this disease experience decreased cysteine amino acid content, which has the capability of forming disulfide bonds (Rogers et al., 2006). It was further concluded that the reduction of UHS KAP proteins were the reason for this decrease (Rogers et al., 2006). Hidrotic ectodermic dysplasia has also been identified as a disease that may be linked

to a lack of KAP production. It is a hereditary hair disease that has been correlated to a loose cortical structure which also includes damage to the cuticle (Fraser & Parry, 2018). Additionally, it was found that there was a decrease in cysteine, proline, and serine, but an increase in tyrosine and phenylamine, indicative of decreased level of KAP expression (Fraser & Parry, 2018). Thus, a decrease in KAPs lead to generally weaker hair.

In addition to hair diseases, there are also several hair treatments known to have detrimental effects that may be related to KAPs. One of the most used hair treatments is bleaching, which involves the use of a cosmetic peroxide-persulphate-based treatment (Grosvenor et al., 2018). Previous studies have demonstrated that this treatment leads to hair fibers that are characterized by degraded melanin granules, a reduced number of cuticle layers, and even decreased definition of the cuticle cell membrane complex (Grosvenor et al., 2018). Moreover, keratin and KAPs have been detected in the leachates of the bleached hair, which demonstrates that the bleaching treatment removes KAPs from the hair (Grosvenor et al., 2018). Furthermore, bleaching treatments are known to oxidize disulfide bonds, hence weakening the strength of the hair, and removing KAPs (Fujii, 2012). Heat damage related to hair straightening has also been identified as a hair treatment that may have a negative relation to the amount of KAPs. A previous study illustrated that excessive exposure to heat damages the hair cortex leading to significant protein loss, which includes KAPs (Lima et al., 2019).

Previously, there have been attempts to derive KAPs from hair using a variety of chemicals. In a study, both KAPs and keratin were successfully obtained from virgin hair (Fujii et al., 2013). The researchers were able to confirm this by conducting a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that illustrated KAPs of molecular weight (M_w) 6-30 kDa present in the filtrate after the “KAPs solution” followed by keratin of M_w 40-65 kDa present in the filtrate after the “Shindai solution” (Fujii et al., 2013). However, the use of thioglycolic acid (TGA) has been explored as an option to be used in the “KAPs solution” to replace dithiothreitol (DTT) due to it being more economical (de Guzman et al., 2015). Thus, even though there have been studies in which KAPs were extracted from hair, there is still room to improve the process to increase functionality and economic circumstances.

In this study, derived KAPs were used as a potential treatment for damaged hair. KAPs have a vital role in the overall structure of hair and are an extremely important element to its overall strength. Additionally, it has been previously identified that several hair diseases and

treatments cause the breakage of disulfide bonds, which KAPs help form. Thus, if the derived KAPs from untreated hair fibers can revitalize the structure of damaged hair, it has the potential to be used as a treatment for previously damaged hair.

Methods

Extraction of KAPs

A "KAPs Solution" was applied to hair fibers for KAPs extraction. Before using this solution, the hair was washed for 60 seconds with distilled water and shampoo and delipidized with a 2:1 mixture of chloroform and methanol. The hair was placed at 30 mg/mL of chloroform and methanol in a shaker at 37 °C. After twenty-four hours, the hair was removed from the shaker and sieve-washed with distilled water. The hair was then placed at 50 mg/mL in "KAPs Solution" containing a 25 mM Tris-HCl at pH 9.5, 25% ethanol (EtOH), 200 mM DTT, and 8 M urea. The hair was placed in the shaker for four days at 37 °C. After extraction, the residual hair was washed with distilled water to remove any remaining chemicals and stored at 4 °C for future use. The filtrate, consisting of filtered soluble cortical KAPs, was then dialyzed against distilled water several times to remove any chemicals. Once the pH of the water for the dialysis was equal to the pH of distilled water, the filtrate was removed from the dialysis, and the proteins present were no longer soluble in water. To attempt to make the extract soluble, several concentrations of sodium hydroxide, DTT, and TGA were applied. The final reduced and solubilized extract was named **ReKAP**.

SDS-PAGE

To determine and confirm the presence of KAPs, a gel electrophoresis analysis (SDS-PAGE) was conducted. Once gels were in place, SDS buffer, which consisted of 0.1% sodium dodecyl sulfate, 25 mM Tris base, and 0.192 M glycine, was added to the tank. Samples were then mixed at a 1:4 ratio using 4x Laemmli buffer (Bio-Rad), heated for five minutes, and centrifuged for five minutes. After preparation, 20 µL of each sample was loaded into each well along with a Precision Plus Protein Dual Color Standards (Bio-Rad) ladder. The power supply was set to 30 mA until samples reached the bottom of the gel. The gel was then removed and placed in a 40% methanol and 10% acetic acid solution for 15 minutes. Afterwards, the gel was

soaked in Coomassie stain (Bio-Rad) overnight. After 24 hours, the gel was rinsed several times with distilled water and viewed using a scanner.

Protein and Melanin Quantification

To quantify protein yield, a DC protein assay (Bio-Rad) was performed using a microplate reader. A 96-well plate was used to load all samples. 5 μ L of each sample was loaded into each well in triplicates. Next, 30 μ L of reagent A followed by 200 μ L of reagent B were loaded. Samples were left to sit for 15 minutes, then absorbance was read in a microplate reader at 750 nm. A standard curve of albumin egg was used, which was made through a series of $\frac{1}{2}$ serial dilutions. Additionally, to determine the amount of melanin in the samples, a Nanodrop spectrophotometer (Thermo-Scientific) was used. During this assay, 2 μ L of the samples were measured at a wavelength of 432 nm. A standard curve using synthetic melanin was employed.

SEM Imaging

A scanning electron microscope (SEM, Quanta 250, FEI / Thermo-Scientific) was used to investigate the residual hair morphology before and after the extraction of KAPs. Samples were dehydrated by submerging in 70%, 90%, and 100% EtOH three times, three minutes each, then left to air-dry overnight in the fume hood. Once fully dried, they were placed on an aluminum stub with double-sided tape, sputter coated with gold (EMS), and imaged at different magnifications.

Damage of Hair

To intentionally damage hair fibers, two different methods were used: bleaching and excessive heat treatment. To conduct bleach damage, normal hair was treated with a 40-V developer (Clairol) and bleach powder (Blond Forte) in a 2:1 ratio for 45 minutes. The bleached hair was then rinsed with distilled water and left to air-dry overnight. For heat damage, normal hair was washed with sodium lauryl ether sulfate, dried with a hairdryer on the highest heat setting, and straightened with an iron at 200°C for 12 seconds. This process was repeated 3 times every 4 minutes, for a total of 12 minutes.

ReKAP Application

To discover the optimal concentration of ReKAP on damaged hair, a dose-response test was conducted at increasing KAPs concentration and were applied to bleach-damaged hair for 30 minutes with shaking. Samples were washed with distilled water and left to air-dry overnight. An ANOVA statistical test was performed, along with a Tukey-Kramer multiple comparison.

Tensile Strength Testing

To determine the hair's strength, the ultimate tensile stress and strain were obtained using the Instron 3345 mechanical tester. Prior to testing, three hair bundles of hair fibers from each group were taped at each end (Figure 1). One end of the hair bundle was placed in the testing grip, while the other end was twisted to form a uniform bundle of hair. This end was then placed on the other testing grip. An image was taken of each bundle with a ruler in the background for analysis of diameter and length in Image J. Samples were tested until failure, and the raw load and displacement data were normalized and used to create stress-strain curves in Microsoft Excel. A Student's t-test was used to determine the statistical significance between each sample's ultimate tensile strength and strain.

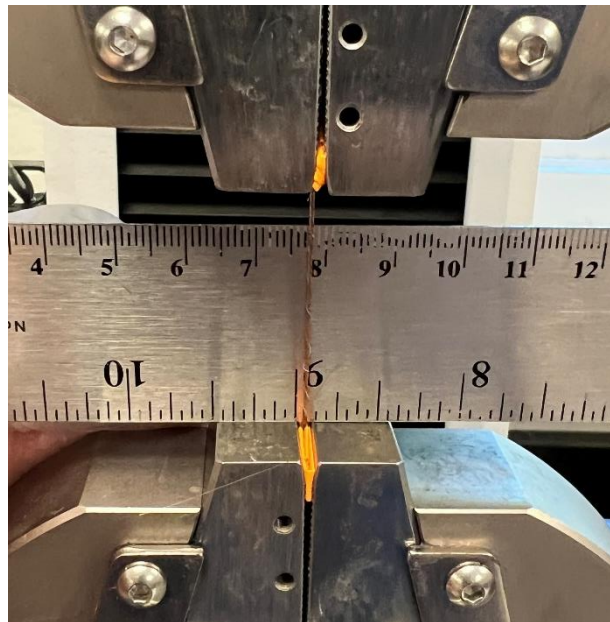


Figure 1: Image taken by student researcher. Set-up of hair being bundled 25 times for tensile testing and then clamped into mechanical tester. Ruler present in background for data analysis.

Results

Extracted KAPs were Low- M_w Proteins

The extraction process yielded proteins with low M_w (mostly at ~ 10 kDa), verifying the presence of KAPs (Figure 2) and no keratin monomer bands based on SDS-PAGE. The sample also included high M_w proteins such as unreduced tightly bound keratin assemblies, keratin-KAP, and KAP-KAP complexes. Additionally, the ReKAP solution demonstrated intensified KAPs bands suggesting that the precipitates from the extract were indeed KAPs that formed disulfide crosslinks generating bigger insoluble molecules. Moreover, in the residual hair, proteins with high M_w were detected, indicating that keratin was not extracted from the hair.

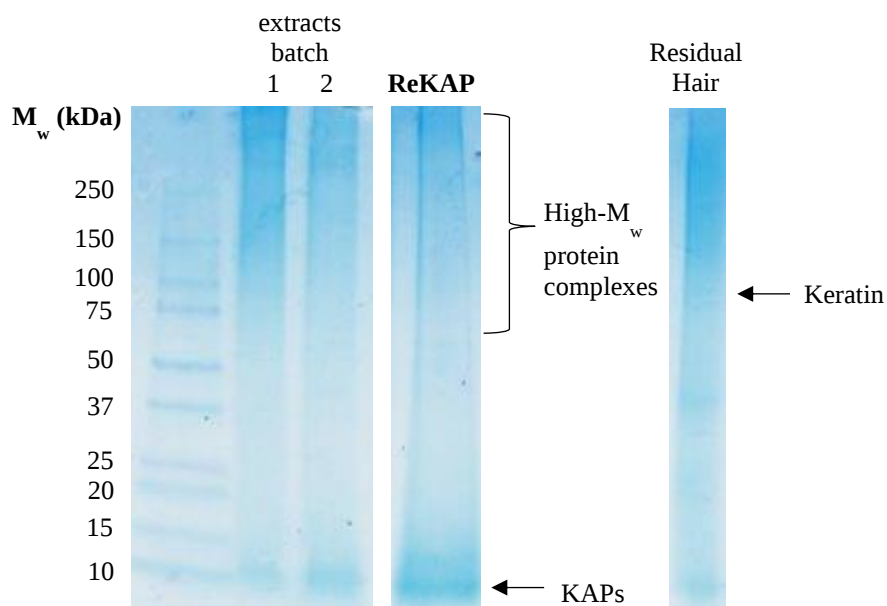


Figure 2: SDS-Page demonstrating the presence of KAPs. Samples included (left to right) KAPs extract 1, KAPs extract 2, ReKAP, and residual hair from KAPs extraction. Low- M_w (10 Kda) KAPs present in ReKAP.

Protein Yield

The total concentration of proteins in the KAPs extract was found to be 3.5 mg/mL (Figure 3), which is a 7% yield from the starting hair mass. The concentration of melanin (indicated by the brownness of the solution) in the sample was found to be 1.5 mg/mL, at a 2.9% yield.

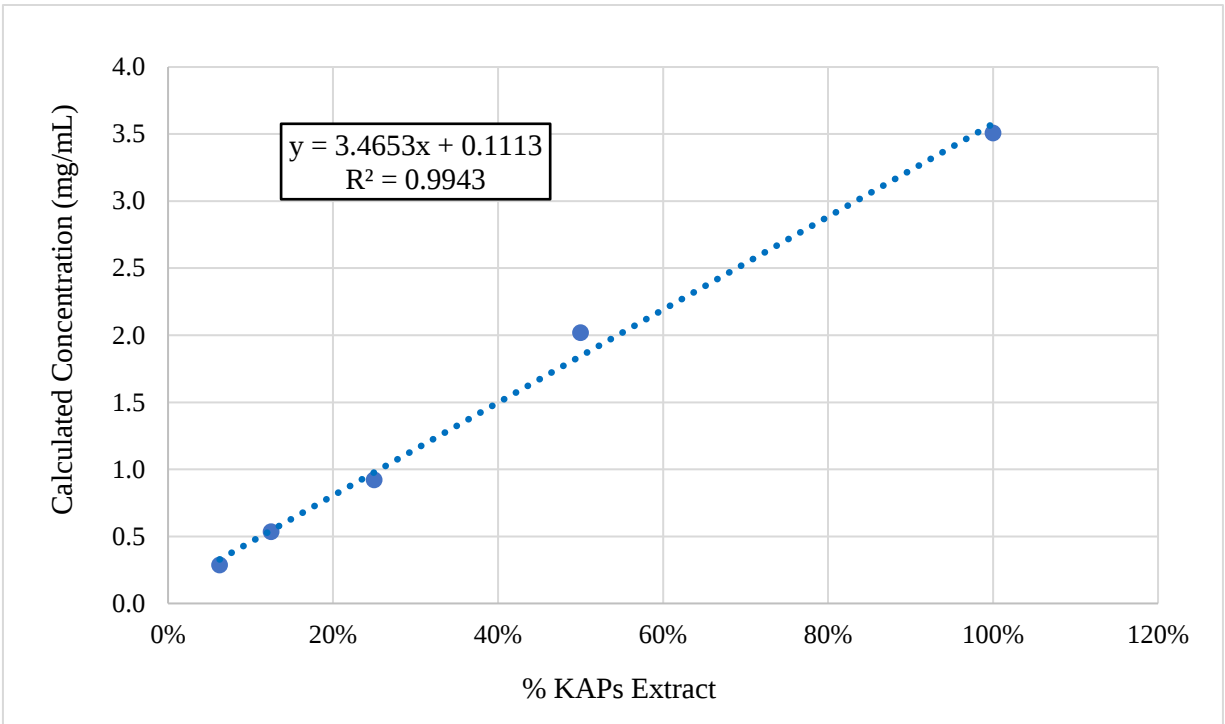


Figure 3. Calculated concentration (mg/mL) of KAPs in KAPs Extract 2 dilutions (1/2 dilution factor). A standard curve of albumin egg was used to determine protein concentrations.

Residual Hair Morphology

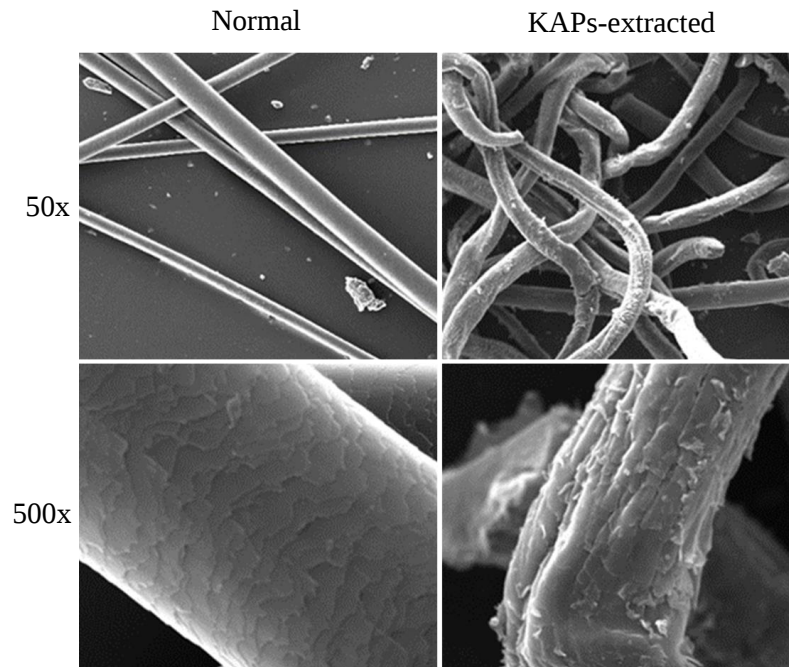


Figure 4. Morphology of untreated hair (left) and KAPs extraction residual hair (right) using scanning electron microscope at 50x and 500x.

SEM-Imaging was also used to display the structure of the hair fibers before and after KAPs Extraction. In the residual hair morphology, significant left-over mass with wrinkled structures and scaly cuticle were still visible in KAPs-Extracted hair when compared to normal hair (Figure 4). This indicates that cortical KAPs and melanin leached out from the loosened outer cuticle during the extraction.

ReKAP Dose-Response Testing

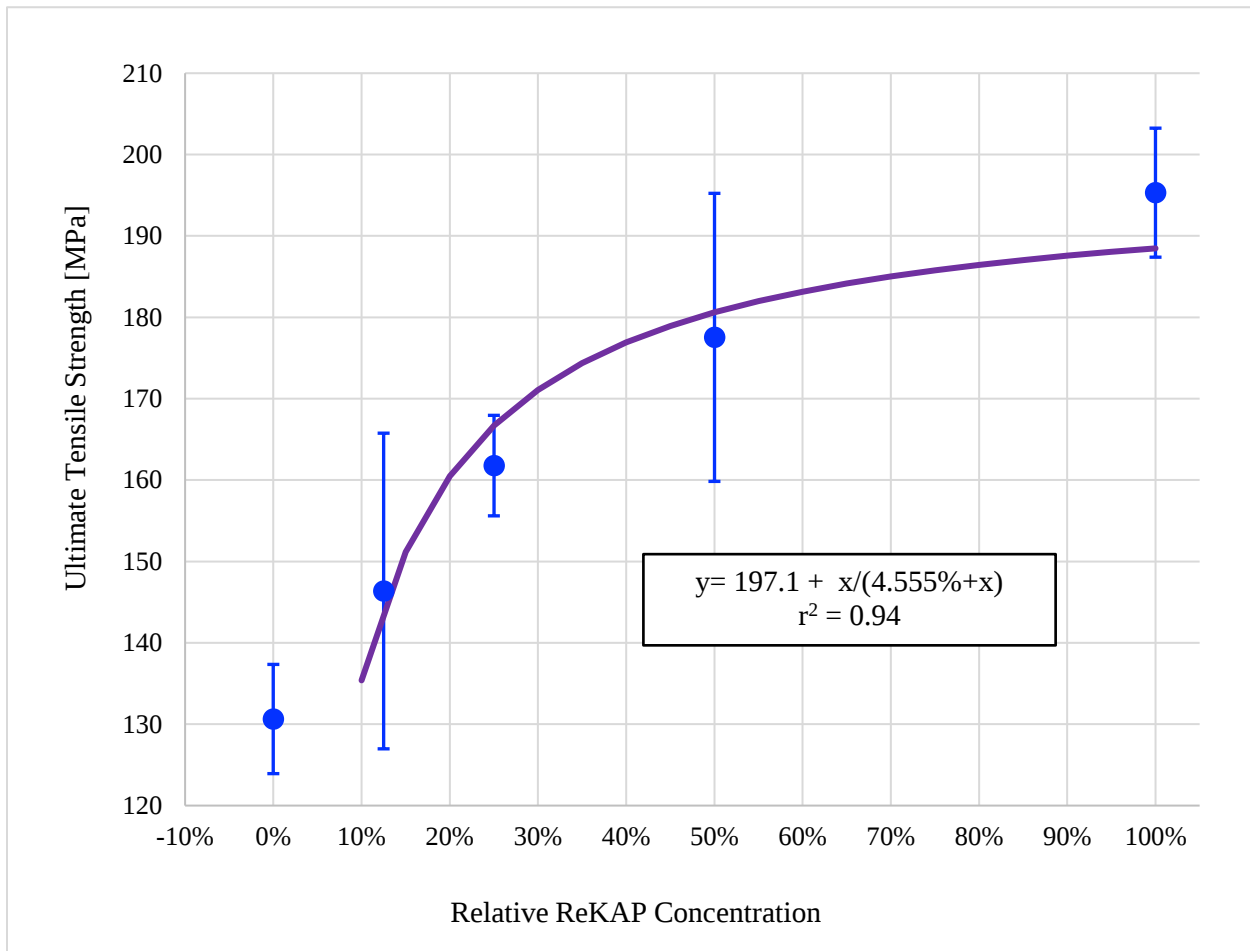


Figure 5. A dose-response curve of ReKAP application in bleached hair. ReKAP was applied at different concentrations for 30 minutes. Statistical significance between all the groups, $p < 0.005$ (ANOVA), and in 1.00 vs 0.00, 1.00 vs 0.25, and 0.50 vs 0.00, $p < 0.05$.

It was found that the ultimate tensile strength (UTS) of bleach-damaged hair without the application of ReKAP was 130.6 MPa (Figure 5). At increasing ReKAP treatment concentration up to 100%, the UTS also increased up to 195.3 MPa (Figure 5), demonstrating a dose-response

relationship of ReKAP application, particularly following a saturation curve function. Moreover, ANOVA determined statistical significance among groups, with a p value of 0.000903. Specifically, there was a statistical significance between the 0 vs. 100% group, $p < 0.05$ (Table 1). Additionally, statistical significance was also observed between other pairs (generally at low vs. high ReKAP).

Tukey HSD Test	
1.000 vs 0.500	Non-significant
1.000 vs 0.250	Non-significant
1.000 vs 0.125	$P < 0.05$
1.000 vs 0.000	$P < 0.05$
0.500 vs 0.250	Non-significant
0.500 vs 0.125	Non-significant
0.500 vs 0.000	$P < 0.05$
0.250 vs 0.125	Non-significant
0.250 vs 0.000	Non-significant
0.125 vs 0.000	Non-significant

Table 1. Tukey HSD Test for ultimate tensile strength for each concentration in the ReKAP application dose-response test.

Effect of ReKAP on Damaged Hair

Tensile testing was also used to illustrate the effects of the application of ReKAP on damaged hair. It was determined that both bleach and heat application statistically significantly, $p < 0.05$, weakened the normal untreated hair, as seen in the drop of UTS from 210.0 MPa to 122.5 and 134.9 MPa, respectively (Figure 6). Application of ReKAP on untreated hair did not statistically increase the hair's strength and its ultimate tensile strain (210 vs. 220.3 MPa and 49.8% vs. 52.4%, respectively, not significant at $p > 0.05$). Additionally, the application of ReKAP on heat-treated hair did not have any significant effects ($p > 0.05$) on the strength (134.5 MPa) or strain (47.9%) of the hair as well.

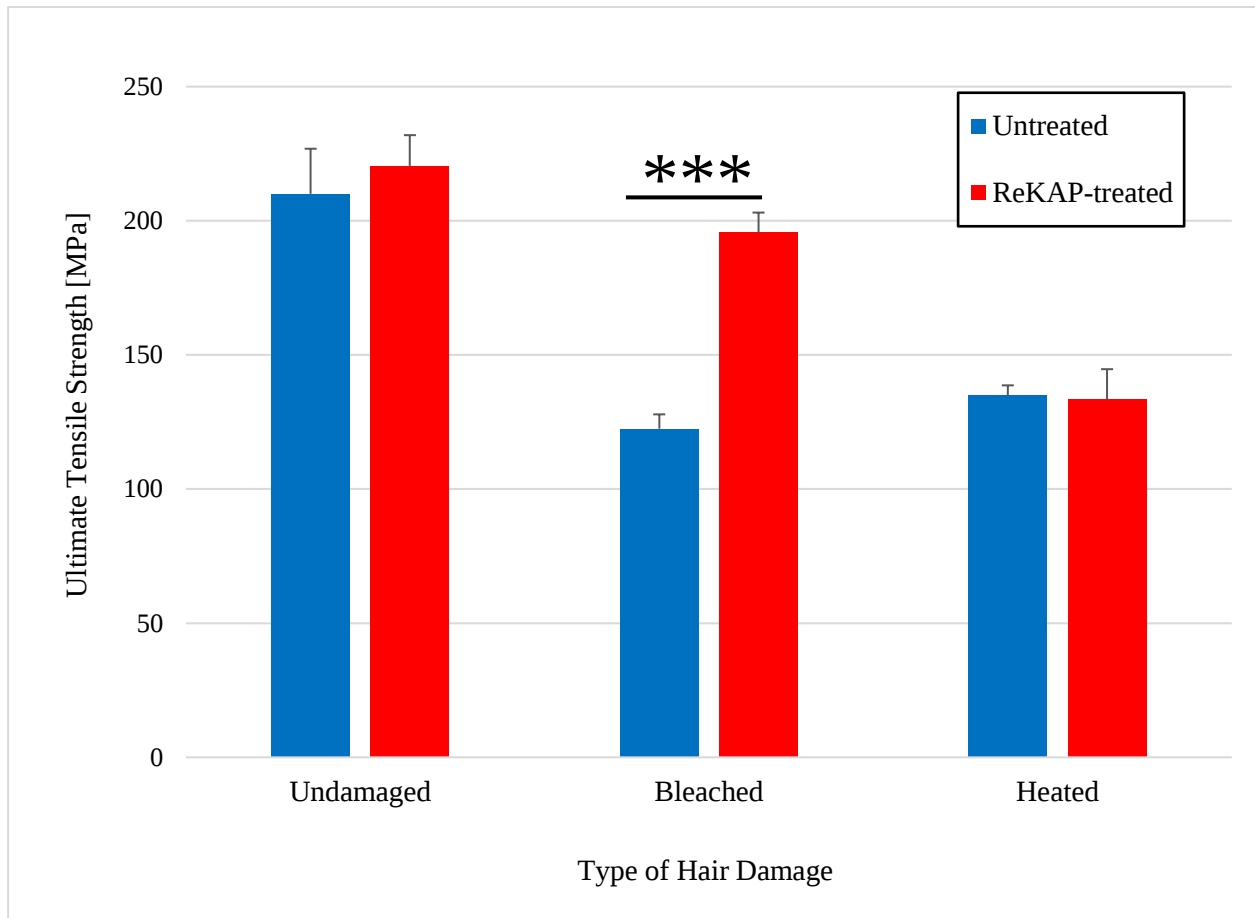


Figure 6. The effect of ReKAP application on ultimate tensile strength on undamaged, bleached, and heated hair, *** $p=0.00016$.

On the other hand, the application of ReKAP did significantly improve the strength of bleached hair. With the application of ReKAP, the ultimate tensile strength increased from 122.5 MPa to 195.6 MPa, which was statistically significant, $p<0.0005$ (Figure 6). The stress-strain curve of multiple replicates clearly showed two distinct curve groups (Figure 7, red = bleached but untreated vs. purple = bleached and treated with ReKAP), indicating an increase in overall strength. However, the ultimate tensile strain did not increase, as without the application of ReKAP, the value was 51.5%, and with the application of ReKAP, the value was 49.4%, which was not significant, $p>0.05$. Thus, even though the application of ReKAP did not significantly improve the strain of the hair, it did in fact rejuvenate the strength to near the level of the untreated hair (Figure 6).

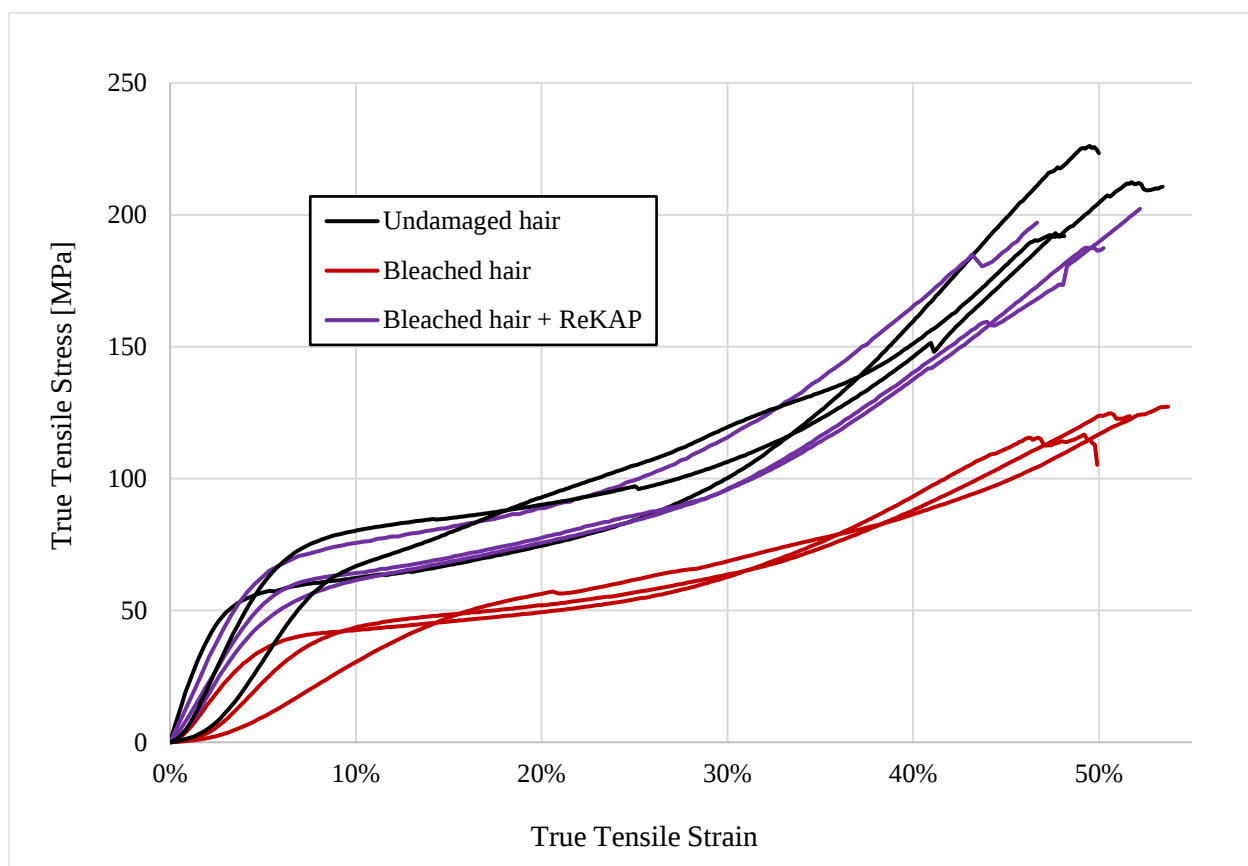


Figure 7. Effect of ReKAP application on the true tensile stress and true tensile strain on bleached hair. Black lines demonstrate undamaged hair, red lines demonstrate bleached hair, and purple lines demonstrate bleached hair with ReKAP application.

Discussion

In this research, the effect of keratin-associated proteins on previously damaged hair was analyzed. Previous research has indicated that KAPs are heavily associated within the formation of hair fibers, which allows them to contribute to the overall strength of the complete hair fiber (Fraser & Parry, 2018). During the formation of a hair fiber, disulfide bonding takes place between keratin intermediate filaments, KIFs, and KAPs alongside disulfide bonds between KAPs and KAPs (Harland et al., 2022). The disulfide bonds which are formed during the process are known to have a significant contribution to the strength of hair. However, in certain hair diseases and treatments, these disulfide bonds are either never formed, or are broken. In this study, it was hypothesized that KAPs, which tend to have a small molecular weight, can be utilized to restore the structure of and strength of damaged hair. This would occur as extracted and reduced KAPs can fit into small pockets of the damaged hair and reform disulfide bonds

with native KIFs and KAPs via oxidation reaction, providing the potential mechanism for success.

In this study, keratin-associated proteins were extracted from healthy hair fibers, and made into a soluble solution, ReKAP. Once this solution was formed, an SDS-PAGE was conducted to view the presence of the proteins. As shown in Figure 1, low molecular weights were detected in the KAPs extract, verifying the presence of KAPs in the sample. Additionally, in the sample containing ReKAP, intensified bands of KAPs were identified, which indicated that the formation of disulfide crosslinks, generating larger molecules. Once the presence of KAPs was detected, the concentration of KAPs in the sample was determined which is demonstrated in Figure 2. It was found that the protein concentration of KAPs in the solution was 3.5 mg/mL, which resulted in a yield of only 7% from the starting hair mass. This may indicate that not all proteins from the cortex were extracted or proteins were lost during the filtration and dialysis processes. Moreover, SEM imaging was conducted to verify the loss of KAPs in the hair fibers during the extraction, which can be demonstrated through Figure 3. In the hair samples before the extraction process, the surface of the hair-fibers was smooth, and the individual hair strands themselves were in a uniform shape and straight. On the other hand, in the samples after the extraction process, the surface was scaly and the structures were heavily wrinkled, indicating that the disulfide bonds that maintain the structure were broken, and ultimately the KAPs were extracted.

Once the ReKAP solution was made, a dose-response test was conducted to determine the effect of increasing concentration of KAPs application. In this test, bleached-hair was used as the control, and it was discovered that the 1.000 concentration of ReKAP had the most statistically significant impact on the strength of bleached-hair, $p < 0.05$, which led to it being used as the concentration for the rest of the study. Additionally, as illustrated in Figure 4, there is a relationship between increasing the concentration of KAPs and the strength of hair, which suggests that a greater amount of KAPs is needed to optimally fill into small pockets of the cortex to restore disulfide bonding. Thus, it was found that ReKAP does improve the strength of hair in a dose-dependent manner.

In Figure 5, the application of ReKAP on different types of damaged hair is represented. It was determined that ReKAP had no statistically significant effect on the ultimate tensile strength of untreated hair or heat-damaged hair as $p > 0.05$. This may be since the hair used in

the study had no significant previous damage, thus there was no disulfide bonds for the KAPs to reform. On the other hand, in heated-hair, most damage is caused by the denaturation or unfolding of protein's tertiary and quaternary structure, but there is no disulfide breakage, so it is likely that no KAPs are lost. However, there was a statistically significant effect on the ultimate tensile strength in bleached-hair, as the p-value presented was 0.00016, which is exhibited in Figure 5. This indicates that since the hair cortex is damaged during the bleaching process, the small-molecule KAPs have the ability to fill-up, seal, and crosslink to restore the damaged hair structure. Thus, due to the promising results found in this study, the application of ReKAP has the potential to revitalize the strength of damaged hair, or possibly prevent the damage from occurring, for millions of people around the world.

Limitations

Due to the limited time in which this study was conducted, only three samples were tested for each group of data collection. Moreover, as the extraction process was not able to be modified in the given time, increased concentrations of KAPs were not able to be tested, which may have limited disulfide bond reformation.

Future Perspectives

In the future, more trials of ReKAP application will be conducted on the groups used in the research to further verify the significance of the results. Moreover, in future studies, the KAPs extraction process will also be optimized, not only to improve efficiency, but also to improve the economic costs due the high cost of chemicals that are used during the process. Additionally, the application of ReKAP will be conducted on a further amount of hair treatments that negatively impact the strength of the hair fiber, and possibly hair diseases to determine potential benefits.

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