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Interaction of polypeptides with hair in different reducing environments

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Abstract

Innovation in product development and cosmetic treatments is essential to meet the demands of the hair care industry. This work studied the influence of reducing environments in the interaction of polypeptides (KP peptide, ELP-KP and BSK recombinant proteins and hydrolyzed keratin) with damaged hair, to recover the hair damage. The reducing conditions resulted from thioglycolic acid and thioanisole addition. The small peptide (KP) demonstrated good penetration into the hair and a higher improve in the fibers' mechanical properties without a reducing environment. In opposition, hydrolyzed keratin and BSK, proteins with high molecular weight and rich in cysteine residues, bounded principally to the cuticle and improved the hair mechanical properties mainly under reducing environments. ELP-KP didn't showed any improvement in fibers' mechanical and thermal properties, probably due to the lower percentage of cysteines and their localization into the protein, reducing the interaction with the hair cysteine residues. A thermal study demonstrated that BSK and KP have a greater increase in the keratin alpha-helix denaturation temperature and enthalpy, respectively, demonstrating the polypeptides stabilizing effect on fibers' secondary structure. These results showed that, not only the reducing environment can influence the products final performance, but also the polypeptides' cysteine content and molecular weight.

Keywords: Polypeptides, keratin, reducing environments, hair, recombinant proteins, mechanical properties, thermal properties.

1. Introduction

Hair treatment is a common life routine and seen as a key component of physical appearance due to its strong psychological and social repercussions [1]. The constant desire to change the morphologic properties of the hair involves continuous innovation in hair cosmetics to develop advanced products according to the consumers' needs [2,3]. To reach the desired hair properties and improve their appearance and self-image, consumers have access to chemical and thermal treatments capable to change hair shape and color [4].

Hair fiber is structured into a cortex region, forming the main bulk of the hair, surrounded by a complex multi-layer cuticle cells [5]. Human hair fiber is mainly composed by proteins, with keratin being the major proteinaceous constituent found in the hair (above 90 %), contributing to its distinctive mechanical strength, durability and flexibility [6]. Keratin contains a significant amount of cysteine, about 18 % of the total amino acid present in its composition. This high cysteine content is crucial for the structural integrity of hair, as it forms disulfide bonds that contribute to hair's strength and shape, and it is also one of the main targets for hair repair products [7].

Hair bleaching is a chemical procedure that causes significant and permanent damage to hair fibers. The oxidation process, primarily using hydrogen peroxide, degrades both melanin and the hair's structural proteins [8]. Bleaching begins at the hair surface, damaging the cuticle and causing it to lift and detach, exposing the underlying cortex. At the molecular level, oxidation affects mainly the amino acid composition, notably cysteine, which is oxidized to cysteic acid. This alteration was confirmed by protein analysis, which demonstrated a relative increase in cysteic acid, glutamic acid, serine, and proline, and a reduction in amino acids such as histidine, tyrosine, and cysteine [9,10]. Oxidation of the lipids associated with the hair fiber is also described in the literature [11]. Structural degradation results in a significant reduction in hair's tensile strength and resistance to breakage, as disulfide bonds, essential for fiber integrity, are broken. The damage also extends to intermediate filament proteins, whose degradation is more pronounced with increasing bleaching treatments, compromising the hair's mechanical strength [12]. In short, bleaching virgin hair results in extensive damage to the cuticle and cortex, with specific degradation of cysteine-rich proteins [13]. These results highlight the importance of techniques and products that minimize oxidative damage to preserve hair health and integrity.

Repairing hair fibers' damage caused by chemical, thermal, and mechanical processes relies on the action of cosmetic agents that act at different levels in the hair structure. These agents do not "heal" the fiber biologically, but rather restructure and protect the fibers' physical, chemical, and mechanical properties [8]. The initial damage manifests itself in the cuticle, with the degradation and lifting of its cells. Superficial repair is achieved through agents that form a protective, cohesive film. Hydrolyzed proteins (e.g., keratin, collagen) and cationic polymers (e.g., polyquaternium-10) adsorb to the surface of the fiber, filling gaps and fissures. This process, mediated by electrostatic interactions and hydrogen bonds, results in the sealing of the cuticle, which reduces water loss, minimizes interfibrillar friction, and restores hair's softness and shine [8,14]. A photoprotective coating of hair against UV radiation is also possible using keratin immobilized into nanotubes [14]. Bleaching and other oxidative treatments compromise the disulfide bonds (-S-S-) that provide strength and integrity to the cortex. Repairing agents, such as bis-aminopropyl diglycol dimaleate, act as "bridge ligands" forming covalent bonds with adjacent

sulfur chains [15]. This mechanism is crucial for restoring broken bonds, resulting in increased tensile strength and elasticity [16]. Furthermore, hair damage results in the loss of vital components such as lipids and amino acids. Replenishment is accomplished by fatty acids (e.g., lauric acid, linoleic acid) and squalane, which resemble the lipids of the hair's surface barrier, penetrate the cortex, and restore hydrophobicity and internal cohesion [17]. Additionally, low-molecular-weight peptides and free amino acids can replenish the lost amino acids, reinforcing the internal protein structure and helping to maintain hydration and manageability [18]. In short, commercialized hair repair products are based on a multifaceted approach: superficial reconstruction of the cuticle, restoration of covalent bonds in the cortex, and replacement of essential molecular components, thus improving the physical and mechanical properties of damaged hair.

A variety of alternative of hair cosmetic formulations containing polypeptides are already described in the literature to be capable to improve hair properties [19,20]. These polypeptides generally have an amino acid composition inspired on the keratin sequence, with high capacity to interact with hair and rich in cysteines to create disulfide bonds. Recombinant DNA techniques have been used in the design of proteins and peptides that mimic the properties of keratins and other structural/functional polypeptides to be incorporated into cosmetic formulations [3,21,22]. Furthermore, recombinant techniques allow the fusion of two or more sequences, creating multifunctional polypeptides with more than one specificity [21,23]. The efficacy of polypeptides, particularly those rich in cysteine, is directly related to their ability to interact with the hair protein structure. However, the interaction and the possible formation of internal bonds, such as disulfide bonds, are complex and depend on several factors [24]. One possible contribution of cysteine-rich polypeptides with molecular weights above 2 kDa is primarily the superficial repair of the cuticle. They act by filling damaged areas, restoring the protective barrier and aesthetic properties of the hair [25]. Hair damage, which often results in negatively charged areas due to the oxidation of cysteine residues, attracts these polypeptides through electrostatic interactions [16]. Adsorption and filling of cuticle fissures are possible mechanisms, resulting in a smoothing effect, reduced friction, and increased shine. The formation of new disulfide bonds (-S-S-) between the applied polypeptide and the proteins of the hair cortex is also possible, especially in the presence of reducing agents that allow the formation of these bonds [8]. It's expected that smaller polypeptides have greater diffusion power, allowing them to penetrate the damaged cuticle more easily, reaching the cortex, and establishing covalent bonds. Several studies involving keratin-based peptides and proteins demonstrate that this interaction can improve hair’s mechanical properties, smoothness and conditioning [23,24,26].

Reducing agents are normally used to break the existing disulfide bonds, allowing the cysteine residues to become available to create new bonds with the cysteine residues in the fibers’ keratin. The reducing agents should act as a "helper" to make the action of the polypeptides more effective. Several reducing agents are used in cosmetics, being thioglycolic acid one of the most used in hair straightening or relaxing treatments. For this reason, it was considered an excellent candidate for use in this study. The cortex of the hair fibers is affected, resulting in the reduction of disulfide bonds (R-S-S-R) present in the hair fiber into separate chains (R-SH HS-R) which allows the reorganization of the hair structure [27–29]. Sometimes additional chemical agents are used to aid the action of certain reducing agents. Due to its

action as an electron donor, we use thioanisole to assist or enhance the action of thioglycolic acid, participating in the breaking of disulfide bonds [30].

In this study, we evaluated the influence of different reducing environments in the interaction of a keratin-based peptide, two recombinant proteins and hydrolyzed keratin with overbleached hair and consequent damaged hair recovery. These polypeptides were incorporated in three ethanolic formulations, at alkaline pH: Formulation 1 - without reducing agents; Formulation 2 - with thioglycolic acid; and Formulation 3 – with thioglycolic acid and thioanisole. Each polypeptide formulation was applied on overbleached hair samples, and the fibers were characterized regarding their performance through mechanical and thermal tests, and the polypeptide localization on hair by fluorescent microscopy analysis.

2. Materials and Methods

2.1. Materials

Natural Asian black hair tresses were purchased from International Hair Importers & Products Inc, USA. The keratin-based peptide (KP) was synthesized by JPT Peptide Technologies GmbH, Germany . The peptide, with a molecular mass of 1599.8 g/mol, was based on the human hair type II cuticular keratin. It includes two cysteine residues, and its sequence is shown in Table 1 [31]. The two recombinant genes designed for the expression of ELP-KP and BSK protein, were synthesized and sub-cloned by GenScript, USA. The polypeptide sequences were added to Table 1. Terrific broth–auto induction medium (TB-AIM) and lysogeny broth (LB) media were purchased from GRiSP, Portugal. Corn steep powder (CSP) was purchased from Roquette, France. Benzyl alcohol, Tween-20 and propylene glycol were sourced from Thermo Scientific, UK). Dimethyl sulfoxide (DMSO), absolute ethanol, sodium chloride and thioglycolic acid were obtained from Fisher Scientific, UK. Fluorescein isothiocyanate (FITC), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), kanamycin monosulfate, Coomassie brilliant blue G-250 and urea were purchased from TCI (Japan), thioanisole, dialysis membrane with 14 kDa cut off, phenylmethanesulfonylfluoride, and other reagents were purchased from Merck (Spain).

Table 1. List of studied polypeptides' sequence**.**

Polypeptide	**Sequence**
KP	GGVCGPSPPCITT
ELP-KP	MVPGVGVPGVGVPGLGVPGVGVPGVGRGGVCGPSPPCITTVPGVGVPGVGVPGLGVPGV GVPGVGRGGVCGPSPPCITTVPGVGVPGVGVPGLGVPGVGVPGVGRGGVCGPSPPCITTV PGVGVPGVGVPGLGVPGVGVPGVGRGGVCGPSPPCITTVPGVGVPGVGVPGLGVPGVGV PGVGRGGVCGPSPPCITTVPGVGVPGVGVPGLGVPGVGVPGVGRGGVCGPSPPCITTVPG VGVPGVGVPGLGVPGVGVPGVGRGGVCGPSPPCITTVPGVGVPGVGVPGLGVPGVGVPG VGRGGVCGPSPPCITTVPGVGVPGVGVPGLGVPGVGVPGVGRGGVCGPSPPCITTVPGVG VPGVGVPGLGVPGVGVPGVGRGGVCGPSPPCITT

	MGHHHHHHSSGLVPRGSHMSCYDLCLPASCSPRPLATSCNEPCYRR
BSK	CWDSTTVIQPPTVVVTLPGPILSSYPQSTTVGSTASAAVGSYLRCCGVPVASGGTRGLGKG ALLCLGGGLGQGQGQGQGQMSCYDLCLPASCSPRPLATSCNEPCYRRCWDSTTVIQPPT VVVTLPGPILSSYPQSTTVGSTASAAVGSYLRCCGVPVASGGTRGLGKGALLCLGGGL

2.2. Expression and purification of ELP-KP and BSK recombinant proteins

Escherichia coli BL21(DE3) containing the pET-28a(+)::BSK vector was used for the expression of BSK in an optimized medium (10 g/L TB-AIM, 30 g/L CSP, 0.934 g/L NaCl, pH 7). *Escherichia coli* BL21(DE3) containing the pET-28a (+)::ELP-KP vector was used for the ELP-KP expression in TB-AIM medium. The culture was grown with initial optical density (600 nm) of 0.1, for 24 h, at 37 °C, 250 rpm. The cells were harvested by centrifugation at 8000 g, at 4 °C, for 5 min.

The purification of the BSK from inclusion bodies was adapted from Pachon *et. al*.[32]. Succinctly, the cells were resuspended in Buffer 1 (2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2 % (v/v) Tween-20, pH 8.0) and were lysed by sonication (60 % amplitude, 3.0 s on plus 9.0 s off for a total of 10 min on) using a 750 Watts vibracell^{TM} Sonics & Materials Inc sonicator. The insoluble fraction obtained after centrifugation for 45 min, at 10000 g, was washed four times using Buffer 1, two times with Buffer 2 (6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, pH 8) and twice with distilled water. The collected pellet was then freeze dried.

For the ELP-KP purification, cells were resuspended in a TE buffer (10 mM Tris, 1 mM EDTA, pH 8), supplemented with 0.1 mM serine protease inhibitor (Phenylmethanesulfonylfluoride), followed by sonication using the same protocol described early for the BSK purification. The soluble and insoluble fraction were separated by centrifugation at 10000 g, at 4 °C, for 45 min. The soluble fraction was heated at 40 °C, for 1 h, and the precipitate collected by centrifugation at 10000 g, for 40 min, at 40 °C. The precipitate was then resuspended in cold water and freeze dried.

2.3. Extraction and purification of hair keratin

Hydrolyzed keratin were obtained from donated human hair from a local barbershop. Pollutants and lipids were removed from hair in accordance with ISO 22716 standards, and the keratin was extracted using a method previously described by Costa et al. (2022) [33]. Succinctly, the hair was incubated in a solution containing 8 M urea, 0.2 M sodium dodecyl sulfate (SDS) and 0.5 M sodium metabisulphite (ratio of 1:10 grams of dried hair to mL of solution). The mixture was heated to 100 °C, for 30 min, following incubation overnight, at 37 °C, with agitation. The extraction solution was centrifugated at 2800 g, for 10 min, and the supernatant filtered with MCE membrane filter (0.45 µm pore size), to remove hair debris. The keratin hydrolysate solution was then dialyzed for 5 days, at room temperature, against distilled water using a dialysis membrane with a 14 kDa cut off.

2.4. Determination of the cysteine content of polypeptides

The percentage of cysteine content for the studied polypeptides was determined based on the KP, ELP-KP and BSK sequences. For the hydrolyzed keratin, the percentage of cysteines was based on previous works from the literature [22].

2.5. Characterization of new polypeptides and hydrolyzed keratin

The polypeptides' size and purity were analyzed by SDS-PAGE. The lyophilized proteins were solubilized (1 mg/mL) in solubilization buffer (6 M urea, 20 mM Tris-HCl pH 8.0, 0.5 M NaCl and 10 mM DTT), mix with loading buffer, and heated at 98 ºC, 5 min. Then, the samples were loaded on 12 % (w/v) acrylamide/bisacrylamide SDS-PAGE and stained with a solution containing Coomassie Brilliant Blue G-250.

2.6. Hair bleaching

Before the bleaching procedure, Asian hair tresses were previously washed with a 0.5 % (w/v) of SDS, for 10 min, following rinsing with current tap water. For each bleaching cycle procedure, the hair tresses were incubated in a bleaching solution (0.1 M of Na_2CO_3/$NaHCO_3$ buffer solution, pH 9.2, with 3 % (w/v) of H_2O_2, at 92-94 °C, for 10 min, following by hair wash with hair commercial shampoo Pantene® Pro-V Classic (1 g of shampoo per 1 g of hair). Bleaching cycles were repeated 7 times to obtain the overbleached (OB) hair samples used in this study as damaged hair model.

2.7. Application of the polypeptide formulations on overbleached Asian hair

All the tested formulations were applied on OB Asian hair. For the applications, hair tresses (300 ± 0.5 mg) were incubated at 25 °C, for 4 min, with 1 mL of 0.01 % (w/v) of the tested keratin-based peptide (KP), BSK and ELP-KP recombinant proteins and hydrolyzed keratin dissolved in 3 different formulations: a) Formulation 1 (F1) composed by ethanolic formulation (20 % (v/v) ethanol, 15 % (v/v) propylene glycol, 0.5 % (v/v) benzyl alcohol), at pH 9; b) Formulation 2 (F2) composed by formulation 1 supplemented with 1 % (v/v) thioglycolic acid, at pH 9; c) Formulation 3 (F3) composed by formulation 2 supplemented with 0.5 % (v/v) thioanisole, at pH 9. After treatment, the hair tresses were washed with water and dried at 25 °C with 50 ± 1 % relative humidity. The concentration of thioglycolic acid and thioanisole used is related to the damage they cause to the hair. Higher concentrations were tested (data not shown), but the hair fiber showed irreversible damage. Therefore, the concentrations were adjusted to those present in the study, which are within the standards of Commission Regulation (EU) 1223/2009.

2.8. Young's modulus of hair fibers treated with the polypeptide formulations

The effect of polypeptide formulations on the mechanical properties of overbleached Asian hair was evaluated using the guidelines outlined in ASTM C1557-14 for fiber tensile testing. A set of forty individual hair fibers (middle section) with low variability on its diameter were selected to be tested and mounted in the tensile grip of the texture analyzer TA.XT plus C from Stable Micro Systems, with a fixed

gauge length of 55 mm. The fibers were stretched until fracture at a rate of 3 mm/min, a trigger of 1 kg, and a preload force of 5 kg From the obtained data was possible to determine the fibers' Young's modulus (stiffness) [21]. The analysis was performed under controlled conditions: 25 ºC and 50 % of relative humidity.

2.9. Thermal properties of hair treated with the polypeptide's formulations

Thermal study of the hair samples treated with the polypeptide formulations in absence or presence of reducing agents were conducted using a differential scanning calorimetry (DSC) instrument (DSC 3500 Sirius, Perkin) with 100 μL gold plated high-pressure crucible (max. pressure: 100 bar). Hair samples with 5-7 mg with 20 μL of distilled water and closed with a 2.4 N torque were analyzed in a temperature range from 25 to 200 °C, at 10 °C/min. Sample analysis was performed using NETZSCH Proteus – Thermal Analysis software (version 8.0.3), where the second derivative was determined, revealing the temperature and enthalpy at which the alpha-keratin in the sample denatured. The DSC instrument was calibrated in a full range of 0 ºC to 420 ºC, heating rate of 10 °C/min, with 4 standards of high purity: indium, bismuth, tin foil, and zinc. Each test group was measured in duplicate, and the mean values were reported.

2.10. Analysis of the hair cuticle using scanning electron microscopy

Samples treated with different formulations were analyzed and morphologically characterized using a desktop scanning electron microscope (SEM) (FlexSEM 1000 II, Hitachi High-Technologies Corporation, Tokyo, Japan). The treated hair samples were mounted on aluminum pin stubs using electrically conductive carbon adhesive tape (PELCO Tabs™). Samples were coated with 2 nm of Au for improved conductivity. Imaging was performed at an accelerating voltage of 5 kV using a secondary electron detector.

2.11. Polypeptides localization in hair by fluorescence microscopy

KP, recombinant proteins and the hydrolyzed keratin were labeled with fluorescein isothiocyanate (FITC) following the manufacturer's instructions (Merck, Spain). The tested proteins, at 2 mg/mL, were dissolved in 0.1 M sodium carbonate buffer, pH 9, and FITC, at 1 mg/mL, was dissolved in DMSO. For each 1 mL of protein solution, 50 mL of FITC solution was added, with gentle and continuous stirring. The reaction was incubated in dark conditions overnight, at 4 °C, to promote the conjugation between the proteins and the probe. To remove the unbound probe, separation using dialysis membranes was performed, for three days against distilled water. The hair fibers treated with the labeled KP, the two recombinant proteins and the hydrolyzed keratin in absence/presence of reducing agent were immobilized in epoxy resin, followed by cross-sectioning of the fibers (20 μm) using a microtome (HistoCore

AUTOCUT R, Leica, Portugal). Cross-sections of hair fiber samples were then analyzed by fluorescence microscopy (OLYMPUS Fluorescence Microscope BX43). All fluorescence images were recorded using 20x objective, 200 ms exposure with the same filter, and brightness intensity, with the software OLYMPUS cell Sens Standard.

2.12. Statistical Analysis

Statistical comparisons were performed using ANOVA with Tukey's multiple comparisons method to identify significant differences between the results obtained. Differences were considered statically significant at p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***), and p-value < 0.0001 (****).

3. Results and Discussion

3.1. Production and purification of recombinant ELP-KP and BSK proteins and extraction of hydrolyzed keratin

The protein BSK was designed based on beta-keratin from *Gallus gallus* and ELP-KP is a fusion protein composed by the KP peptide with an elastin-like polypeptide (ELP). The addition of the KP peptide to the ELP sequence was to increase the number of cysteines present in the protein, increasing the probability to form disulfide bonds with the hair and its affinity to the fiber. They were produced in *E. coli* BL21(DE3). ELP-KP expression was carried out in TB-AIM medium at 37 ℃ with shaking at 250 rpm, while BSK was expressed in an optimized medium composed of 10 g/L TB-AIM, 30 g/L CSP, and 0.934 g/L NaCl, adjusted to pH 7.0. The BSK purification was carried out via inclusion bodies isolation while the ELP-KP purification was performed by inverse temperature transition at 40 ºC. The hydrolyzed keratin was extracted from hair using an alkaline hydrolysis method. The purity of the three polypeptides was assessed by SDS-Page and is represented in Figure 1. To note that none of the tested molecules were purified via highly specific chromatographic methods and, as such, some degree of impurities is expected. The molecular weights of ELP-KP and BSK proteins were approximately 34.0 and 22.8 kDa, respectively, as evidenced by the distinct bands observed at those positions on the SDS-PAGE gel. ELPs are known to have a smear-like behavior on SDS-PAGE that can be seen on the gel, especially bellow the expected molecular weight [34]. Bands that appear above the expected molecular weight could be either contaminants or possible structures resultant from interaction between the high cysteine content of these proteins. Relatively to the hydrolyzed keratin sample, it is possible to observe in Figure 1 the typical pattern of diffuse bands in a smear-like behavior through the gel, with two intense bands are identified in the region of approximately 40-70 kDa [35]. A study carried by Cruz et al. (2017) performed an analysis of hydrolyzed keratin extracted using the same method, where the bands and size of the protein were identified [36]. It was observed a band between 40 and 60 kDa that correspond primarily to microfibrillar keratins, and a band of approximately 68 kDa that corresponds to type II keratins [22,37]. KP, due to its low molecular weight (1.2 kDa), was analyzed by HPLC where a purity degree of 72.4 % was obtained (data not shown).

Figure 1. 12 % (w/v) acrylamide/bisacrylamide SDS-PAGE of hydrolyzed keratin, BSK and ELP-KP polypeptides (1 mg/mL). MW: GRS Protein Marker Blue (GRiSP, Portugal).

3.2. Mechanical resistance of hair treated with polypeptide's formulations

Proteins, peptides and hydrolyzed proteins are currently used for several procedures in hair cosmetics, namely for hair strengthening, thermal protection and modulation [38,39]. KP, ELP-KP, BSK and the hydrolyzed keratin were studied under reducing environments by the addition of thioglycolic acid or thioglycolic acid and thioanisole to the ethanolic formulation, at pH 9. Since these chemical agents are described as interveners in chemical bonds reduction [2,16,40], it is expected that these agents can influence the polypeptides' interaction with hair fibers by affecting intra and intermolecular interactions. A previous study have already shown that KP and hydrolyzed keratin can, partially, recover the mechanical and thermal properties of overbleach hair [41], however, the effect of a reducing environment during the polypeptide's application was not evaluated.

In the context of hair research, the evaluation of fiber tensile strength represents a critical parameter, as it is closely associated with the structural integrity of the cortex, particularly its disulfide bonds content. Through extension tests, the stress versus strain behavior of the hair fibers is obtained considering the force applied to the hair per unit area and the fibers' deformation [42]. Young's modulus is a mechanical property related to both tensile strength and fiber strain, providing information about the material stiffness [38]. It is a crucial indicator of hair integrity, that could be related with the intermolecular interaction between applied polypeptides and the keratin from hair, resulting in an enhancement in fibers' stiffness. Figure 2 shows the Young's modulus characterization of the hair tresses after application of the different formulations containing KP, ELP-KP, BSK and the hydrolyzed keratin under different reducing environments. The stress versus strain graph for the OBQ7 Asian hair treated with the studied polypeptides under the three reducing environments, at pH 9, is shown in the Supplementary Material (Figure S1).

Figure 2. Young's modulus of overbleached hair tresses treated with 0.01% (w/v) of keratin-based peptide (KP), ELP-KP or BSK recombinant proteins or hydrolyzed keratin under 3 different formulations. Formulation 1 composed by 20 % (v/v) ethanol, 15 % (v/v) propylene glycol, 0.5 % (v/v) benzyl alcohol, at pH 9; b) Formulation 2 composed by formulation 1 supplemented with 1 % (v/v) thioglycolic acid, at pH 9; c) Formulation 3 composed by formulation 2 supplemented with 0.5 % (v/v) thioanisole, at pH 9. The fibers' Young's modulus was calculated based on the average of 40 single hair fibers, with the bar corresponding to the standard error. Grouping information using Tukey's multiple comparisons consider differences statistically significant at p-value < 0.05 (*), at p-value < 0.01 (**), at p-value < 0.001 (***), and at p-value < 0.0001 (****).

The tested polypeptides have a high content of cysteine residues in their sequence (KP contains 15.4 % (2 cysteines), ELP-KP 5.1 % (20 cysteines), BSK 8.0 % (18 cysteines) and hydrolyzed keratin 7-20 % (17 to 32 cysteines)) what can influence the formation of covalent interactions with hair proteins [22,43,44]. This kind of interaction are deeply related with the improvement of mechanical properties in

hair fibers [3]. Analyzing the effect of the reducing environments, an improvement of 25 % and 9 % in the fibers' Young's modulus was observed for the BSK recombinant protein when prepared in Formulation 2 and Formulation 3, respectively, and compared with the control formulation, without any reducing agent. Molecules with higher molecular weight, such as BSK protein, have lower probability to penetrate within the hair and form bonds with cysteine residues from the keratins present in the cortex region. However, the relaxation of the hair fiber using reducing environments, could improve the probability for an interaction by having more reduced cystine residues (cysteines) available for interaction. Besides the molecular weight, the amino acid composition around free cysteine residues can also influence the formation of disulfide bonds. In the case of BSK, the protein contains aromatic amino acids (8 tyrosine and 2 tryptophan) and a high percentage of polar amino acids that are described in the literature as a favorable environment for the formation of disulfide bonds between free cysteines from the polypeptide sequence and the hair fiber [45]. For the hydrolyzed keratin, an improvement of 33 % and 29 %, when in the presence of Formulation 2 and Formulation 3, respectively, was found in comparison with Formulation 1. The extent of improvement between non-reducing and reducing environment was even higher for this sample, although the differences between both reducing formulations were not significative. The improvement of this protein was mainly due to the presence of the reducing agent thioglycolic acid, and the high content of cystines present in this protein. As it is a hydrolysate derived from the hair fiber, it can show a greater affinity to bind to the hair, especially to bleached hair since it contains a higher percentage of available cysteine residues [46]. This protein will reform the hair's covalent bonds, considering that, compared to other proteins, it contains a high level of free cysteines that can bind covalently to the hair fiber. This factor will increase the fiber's Young's modulus, allowing the fiber structure to regain its mechanical strength. Also, since it contains polypeptides with a high variability in molecular weights, the hydrolyzed keratin can form a film around the hair fiber, as can be observed in the SEM images from Figure S2. Hair samples treated with hydrolyzed keratin present a smoother and more uniform surface comparatively to the remain samples. The Young's modulus for hair treated with ELP-KP showed a non-significant improvement over the three formulations tested. This results may suggest that the action of this polypeptide on hair may not occur due to the amorphous structure of the elastin blocks of ELP-KP that may affect the availability of cysteine residues to interact with the hair fiber, and the action of the reducing agents that may affect negatively the redox state of the protein [47]. It should be noted that ELP-KP has a sequence without aromatic amino acids what may difficult the formation of disulfide bonds with the cysteines present in the hair fiber [45]. Studies demonstrate that the KP peptide plays a fundamental role in hair recovery. The mechanism of action of KP is associated with its ability to interact with structural proteins of the hair fiber, favoring the reorganization of keratin chains and strengthening of the intercellular matrix. KP works by binding to the hair and promoting bond integrity, resulting in a safer and less aggressive recovery [3,21,31]. This translates into a significant improvement in the mechanical resistance 39 % when compared to overbleached hair in this study, optimizing hair treatments and reducing the potential damage often observed with the use of reducing compounds [3]. However, the use of reducing agents in the formulation with KP did not demonstrate an improvement in the action of the peptide on the mechanical properties. KP obtained a decrease of 19 % for Formulation 2 and 14 % for Formulation 3. This may be related to the

fact that the use of agents such as thioglycolic acid and thioanisole may alter the redox potential of the KP peptide, influencing its structural and functional properties, especially due to the presence of cysteine residues [48]. When observed by SEM (Figure S2), no deposition of KP was observed on the overbleached hair fibers, demonstrating the capacity of this peptide to penetrate the hair fiber, reaching the cortex, where it can form new chemical bonds.

The use of reducing agents promoted a mechanical improvement in the fibers treated with BSK and hydrolyzed keratin but exerted a different effect in ELP-KP and KP. With the action of thioglycolic acid and thioglycolic acid plus thioanisole, the proteins from the fiber ended up with free cysteine residues, both in the cortex and in the cuticle, increasing the potential bonding points to connect with BSK and hydrolyzed keratin. We can affirm that small peptides with low cysteine content can penetrate and exert effective action on the hair; this cannot be verified when the peptide has a high molecular weight. The effect of polypeptides' molecular weight on the effectiveness for hair penetration was already reported in Carvalho et al. (2025), where three peptides with molecular weight below 3000 Da penetrate more deeply through the cuticle reaching the cortex, comparatively to polypeptides with higher molecular weight [49]. However, if the cysteine content is high, the action of reducing environments aids the action on the hair, promoting improvement in fiber properties.

3.3. Thermal properties of hair treated with polypeptide's formulations

Wet-DSC was used to determine the potential of each polypeptide to prevent or delay the denaturation of keratin alpha-helix present in the hair fiber. Studies carried out by Hally and Snaith (1967) in moist hair fibers demonstrated the presence of an endothermic peak with a maximum temperature of approximately 135-150 °C [53], a result attributed to the loss of structural organization of keratin fibers due to the denaturation of keratin alpha-helix structure [54,55]. These studies complement the structural effect polypeptides could exert in the resistance of hair. Figure 3 shows the keratin alpha-helix denaturation temperature and enthalpy in hair tresses treated with KP, ELP-KP, BSK and hydrolyzed keratin dissolved in ethanolic formulation and obtained by wet-DSC. It was not possible to verify the existence of a keratin alpha-helix denaturation peak for hair fibers when the polypeptides were applied in reducing environments with thioglycolic acid and with thioglycolic acid plus thioanisole. Example of wet-DSC graph of polypeptides in formulation 1, 2 and 3 on Supplementary Material (Figure S2). This result is, much probably, related with the humid environment where the was carried out, resulting in the disorganization of the hair, losing the alpha-keratin denaturation peak.

Figure 3. Keratin alpha-helix denaturation temperature and enthalpy of overbleached hair tresses after application of 0.01 % (w/v) of KP, ELP-KP or BSK recombinant proteins and hydrolyzed keratin dissolved in Formulation 1 composed by ethanolic formulation, pH 9. Values plotted in the figure corresponds to the average of two independent measurements, with the bars corresponding to the standard error. Grouping information using Tukey's multiple comparisons consider differences statistically significant at p-value < 0.01 (**), p-value < 0.001 (***) and p-value < 0.0001 (****) (Table S1 and Table S2).

The keratin alpha-helix denaturation temperature ranged from 137.9 °C to 143.2 °C in the tested samples [56]. Although all samples had an increase in denaturation temperature, it was observed a significative increase in the endothermic peak temperature value for hair tresses treated with the BSK protein. Since this protein is rich in cysteine residues and have aromatic and polar amino acids, BSK could form a higher number of disulfide bonds with the cysteines from the hair, stabilizing the keratin alpha-helix structure. For this reason, a higher temperature will be needed to breaks this new bonds [57,58]. Regarding the denaturation enthalpy of the keratin alpha helix, it was significantly better for all samples. This demonstrated that the integrity of the alpha-helical structure was partially restored, demonstrating that the action of the polypeptides was effective in improving the resistance of the fibers against thermal damage. This improvement may be related to the content of amino acids present in the polypeptides, which are rich in glycine and essentially in cysteine, increasing the number of disulfide bonds in the hair fiber [59]. The higher improve in denaturation enthalpy was obtained for KP, which is a peptide with high affinity to hair, having a good capacity to restore existing damage, as previously demonstrated in Cruz et al. (2017) [3]. Along with the fact that it has a high affinity with the fiber, KP is a peptide with low molecular weight what facilitates its penetration into the hair fiber, and have a high cysteine content which allows the formation of disulfide bonds with the free cysteines of the hair fiber [31]. These factors provided an increase in the enthalpy value, if the peptide managed to reach the fiber cortex in greater quantity, promoting greater protection against keratin denaturation.

No denaturation peaks were observed in hair samples where the polypeptides were applied in reducing environments. The literature shows that the cortex is essentially composed of alpha-helical intermediate filaments (IF) immersed in amorphous material [60]. Considering that the thermal test was performed in a humid environment, the cortex undergoes a phase change, increasing the disorganization of the hair fiber matrix [61]. With this disorganization and the action of reducing environments, the alpha-keratin denaturation peak was not noticeable. Although the action of the peptides under reducing environments was proved effective on the stress-strain testes, when the thermal analysis was performed in humid environment a matrix oscillation occurs. We believe that while fiber denaturation occurs, the process is spread throughout a wider range of temperature, ending up not being perceptible as a peak in the spectrum. A similar behavior was already reported by Istrate et al. (2009) where they observed a shift in the keratin denaturation peak to 100 ºC for virgin Caucasian hair and a reduction in the enthalpy values [62].

3.4. Interaction of the polypeptide in the overbleached hair fiber

The polypeptides in different reducing environments were analyzed regarding their ability to interact with the overbleached hair fiber using fluorescence microscopy. Penetration into the hair fiber does not only come from the state of degradation of the cuticle and cortex, but also from the molecular weight of each polypeptide [49]. Analyzing the Figure 4, it is possible to observe the polypeptides interaction with overbleached hair's cuticle, cortex and, in some cases, the medulla.

Figure 4. Fluorescent microscopy images of transversal cuts of overbleached Asian hair treated with keratin-based peptide (KP), ELP-KP or BSK recombinant proteins or hydrolyzed keratin at 0.01 % (w/v) in Formulation 1, Formulation 2 or Formulation 3. For each sample, 1 mL of solution was applied. Scale bar is 50 μm.

This approach was performed to confirm the localization/penetration of polypeptides in overbleached hair when applied in different reducing environments. Although the extent of the damage resulting from the overbleached treatment can affect the polypeptides' binding capacity, the main properties responsible for the polypeptides' interaction are their size and their amino acid composition.

The KP peptide demonstrated a good penetration profile into the bleached hair fiber, which was the best result of the tested hair samples in the absence of reducing agents, as demonstrated by the fluorescence intensity graph (Figure S3). This peptide has high capacity to interact with hair fiber due to the formation of hydrogen and disulfide bonds during the application [3]. These results are in accordance with the Youngs' modulus data since KP, without the action of reducing agents, obtained a significant result in the recovery of mechanical damage and protection by thermal denaturation. The ELP-KP showed to localize in the cuticle in the absence of reducing agents but with low intensity. This suggests that ELP-KP does not interact with hair, probably because there are no disulfide bonds between the peptide and the hair, or even because the redox state of the protein may have been affected using thioglycolic acid and thioanisole.

In the presence of reducing agents, BSK and hydrolyzed keratin showed good fluorescence when applied in a reducing environment. With Formulation 2, hydrolyzed keratin and BSK showed a high fluorescence, with the proteins located predominantly in the cuticle. The same occurred with hydrolyzed keratin in Formulation 3, where the protein is present primarily in the hair fiber's cuticle but also present fluorescent spots in the hair cortex, that could be lower molecular weight peptides present in the hydrolyzed keratin.

The fluorescence data helped to demonstrate the preferential location of each polypeptide in the hair fiber, in the presence or absence of reducing agents. In short, we can see that the KP peptide presents a more uniform fluorescence in the absence of reducing agents than the other samples, being intensely localized in the cortex, corroborating the results of Young's modulus and wet-DSC. In the presence of reducing agents, the hydrolyzed keratin demonstrated a more intense fluorescence in the cuticle of the hair fiber, suggesting that this protein may form a film around the fiber [25], justifying the Young's modulus result, both in Formulation 2 and in Formulation 3. Like the hydrolyzed keratin, BSK presented a more intense fluorescence in the fibers' cuticle, justifying the promising results in the mechanical test. Both polypeptides presented a similar behavior when applied in a reducing environment.

4. Conclusions

To understand the effect of reducing environments when polypeptides are applied to hair, we tested different properties of hair fiber after the application of KP, ELP-KP, BSK and hydrolyzed keratin, together with thioglycolic acid or/and thioanisole in the formulation. Bleached hair samples were used in this study as a damaged hair model. These environments helped to understand the best performance

conditions of the polypeptides, as well as to understand whether the intrinsic characteristics of the polypeptides (size and amino acid content) could influence their interaction with hair.

We observed that the polypeptides application under different reducing environments promoted different effects on the hair. KP, due to its small size and low number of cysteine residues (15.4 %), presented the best recovery result in the mechanical test in the absence of reducing agents, with the peptide being in the fibers' cuticle and cortex. Regarding the use of reducing environments, BSK and hydrolyzed keratin provided the best mechanical recovery results in the presence of thioglycolic acid. With the use of thioglycolic acid and thioanisole, hydrolyzed keratin presented the best results. By fluorescence and scanning electron microscopy, the BSK and hydrolyzed keratin demonstrated higher presence in the hair cuticle, possibly due to the higher size that difficult the polypeptides penetration in the fiber. Since both polypeptides were able to form an external layer around the fibers, both improved the fibers' mechanical properties.

In the thermal test, it was only possible to obtain data regarding the denaturation peaks of alpha-keratin in the samples without reducing agents, due to the organized state of the cortex matrix. In terms of denaturation temperature, BSK was the peptide that presented the higher temperature increase, while KP presented the highest enthalpy, demonstrating that more energy is required for keratin alpha-helix denaturation. ELP-KP did not present prominent fluorescence when compared to the other samples, regardless of the environment in which it was tested, corroborating the mechanical and thermal results.

In short, this study demonstrated that the interaction of polypeptides with the hair does not depend solely on the reducing environments. The improvement in hair characteristics was proven through mechanical and thermal tests, highlighting the importance of amino acids composition and the size of each polypeptide in improving the fibers' final properties. Under the most favorable conditions for each polypeptide, they can be used as active ingredients in cosmetic formulations, replacing chemical agents that are harmful to the consumer and the environment.

Author Contributions: CRedIT

José Pedro Carvalho: Methodology, Validation, Formal analysis, Investigation and Writing—original draft.

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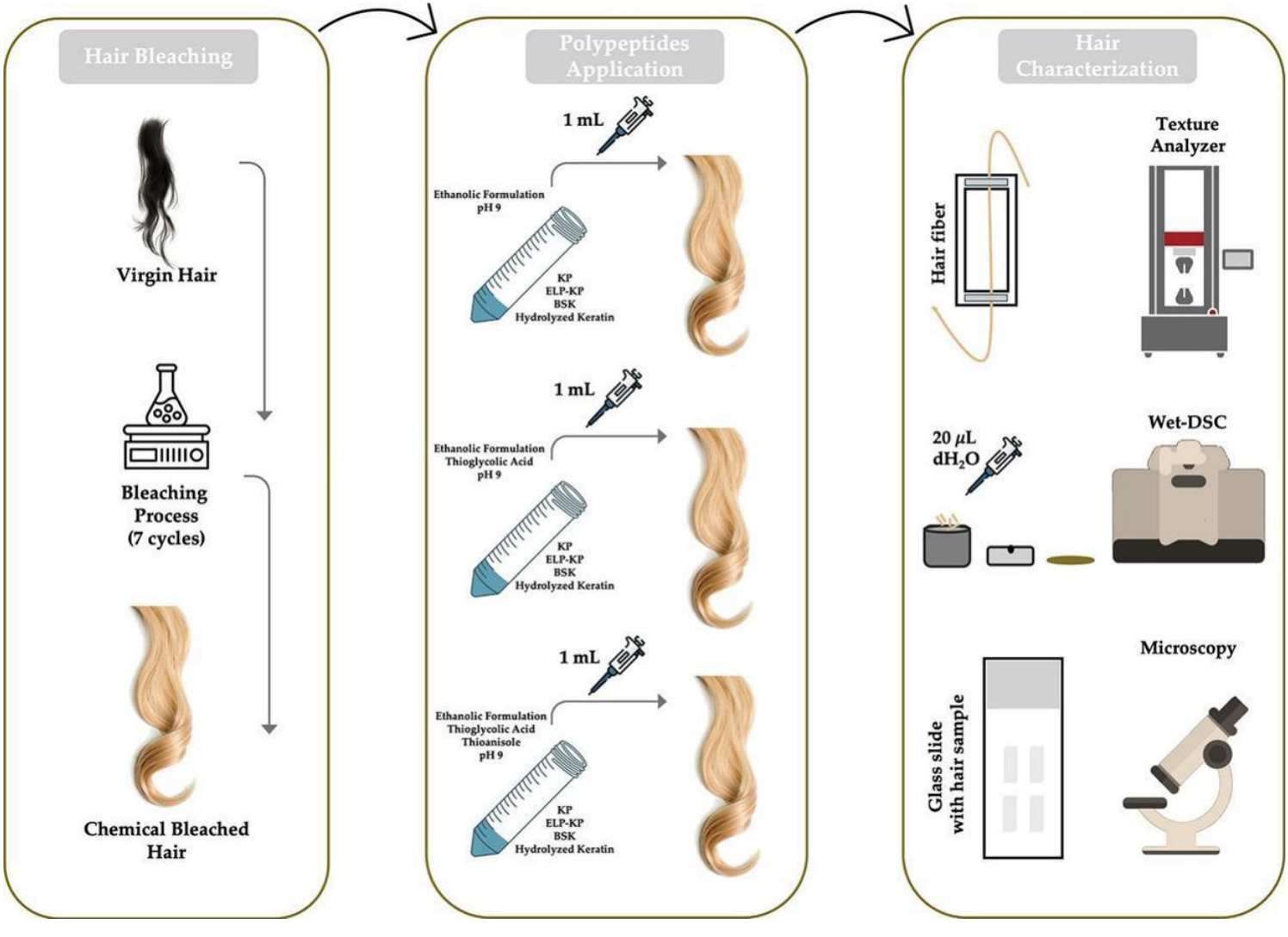
Graphical abstract

Highlights

- Reducing environments impact the polypeptides potential for hair applications.
- KP, in the absence of reducing agents, penetrates and restores the hair properties.
- Larger polypeptides, under reducing environments, bound preferentially to cuticle.
- Reducing environments influenced more the polypeptides with high cysteine content.



Graphics Abstract

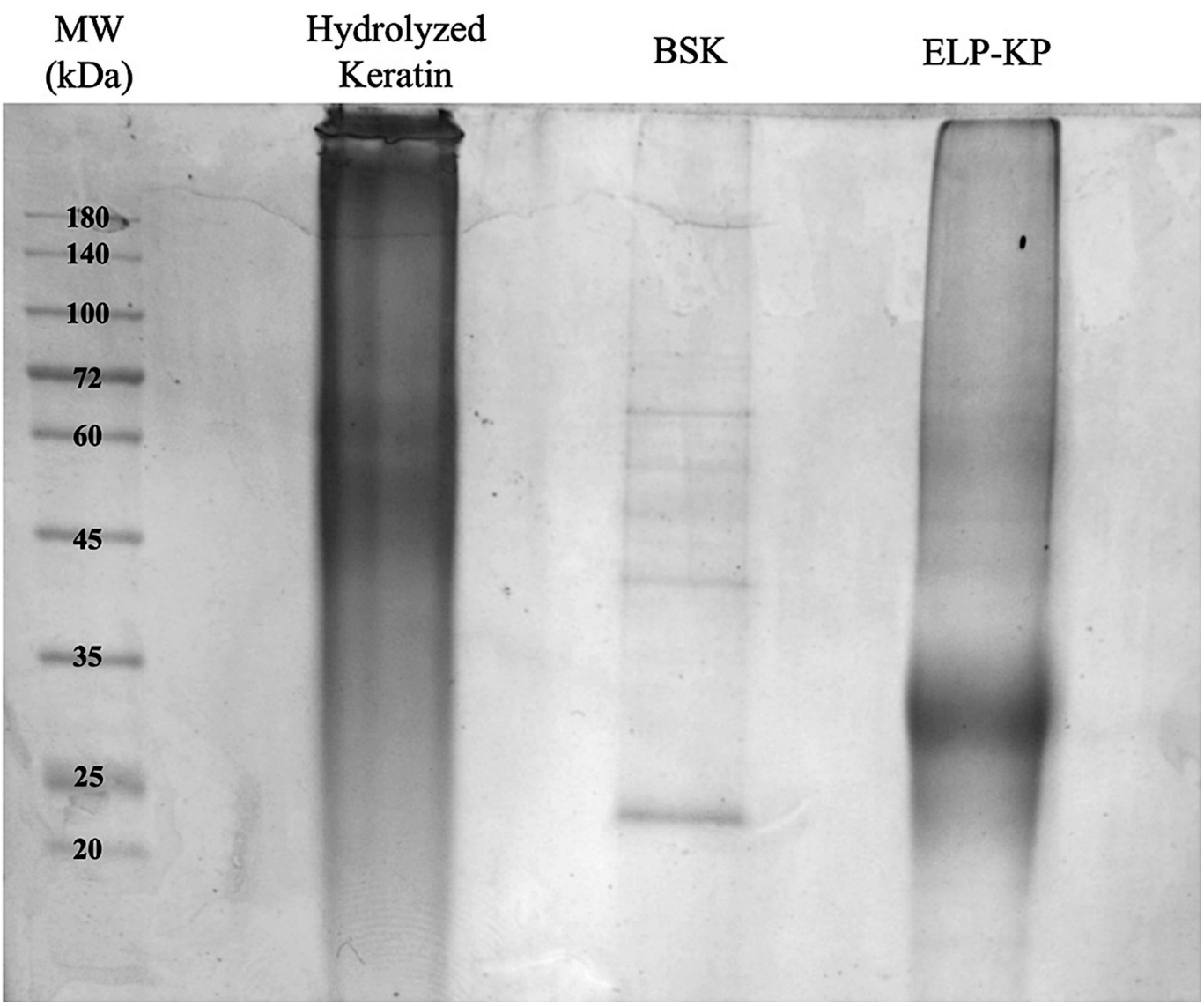

MW
(kDa)
Hydrolyzed
Keratin
BSK
ELP-KP
180
140
100
72
60
45
35
25
20

Figure 1

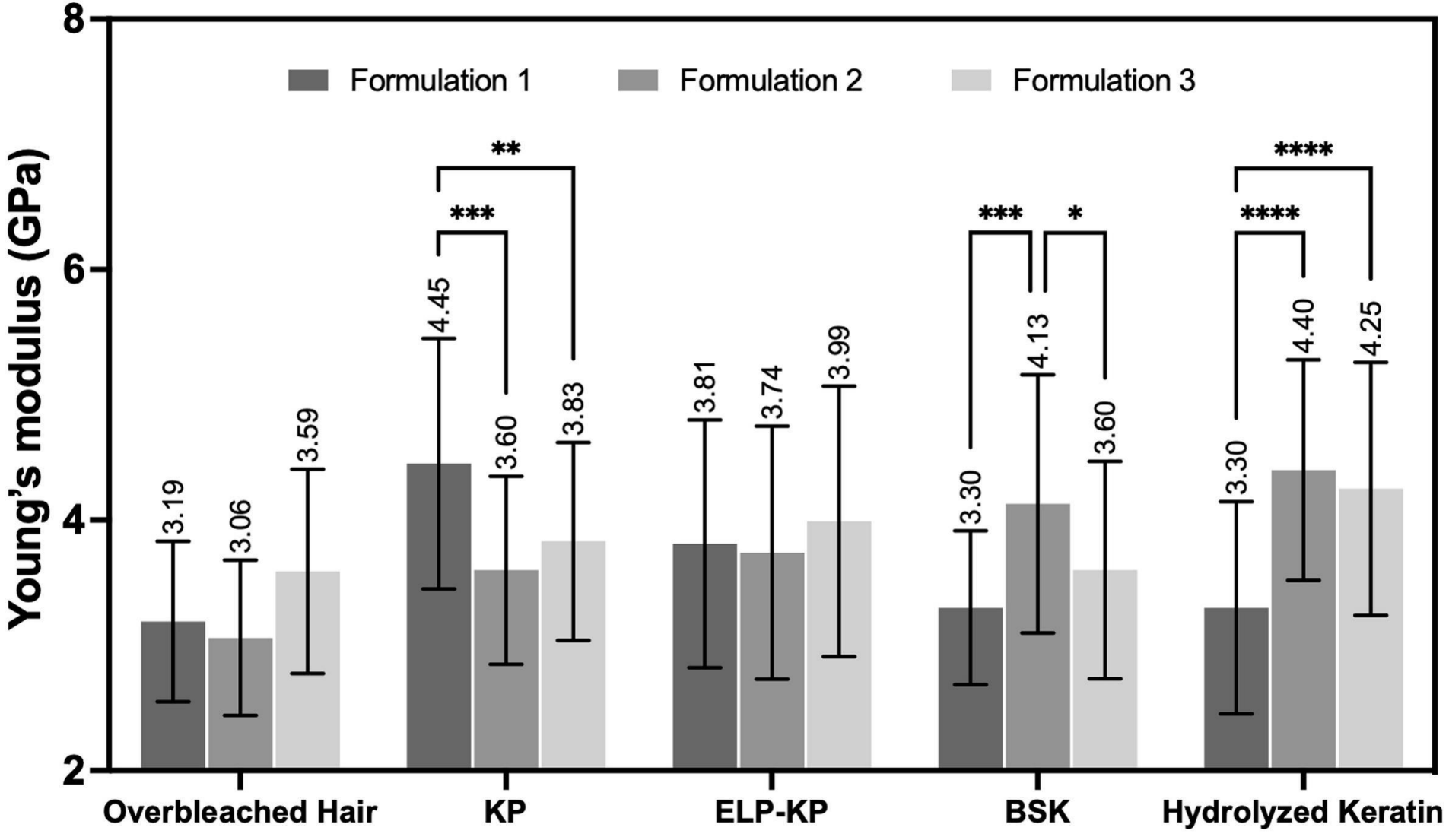

Formulation 1
Formulation 2
Formulation 3
Young's modulus (GPa)
8
6
4
2
3.19
3.06
3.59
4.45
3.60
3.83
3.81
3.74
3.99
3.30
4.13
3.60
3.30
4.40
4.25
**
***
***
*
****
****
Overbleached Hair
KP
ELP-KP
BSK
Hydrolyzed Keratin

Figure 2

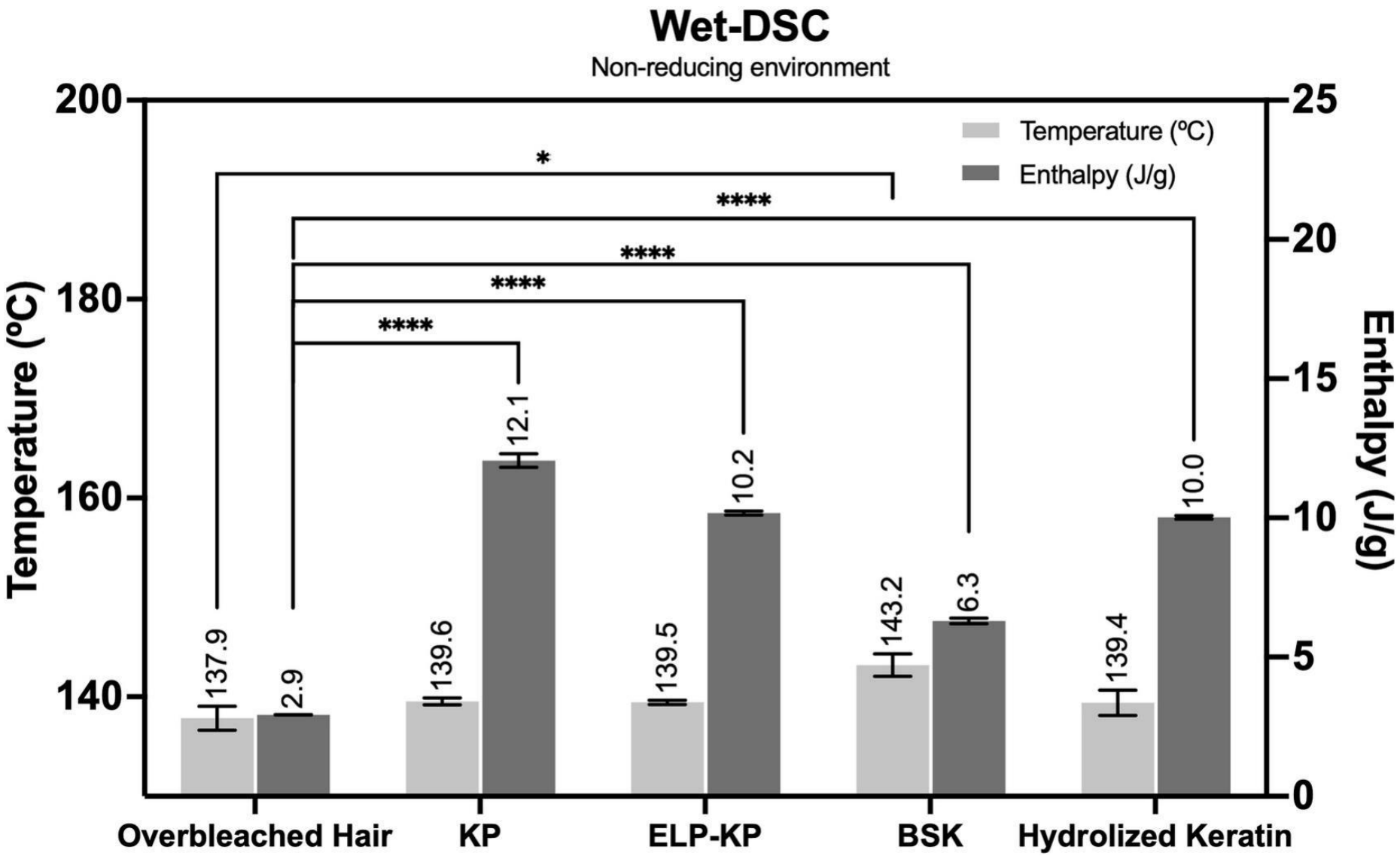

Wet-DSC
Non-reducing environment
Temperature (ºC)
Enthalpy (J/g)
200
180
160
140
25
20
15
10
5
0
*
****
****
****
****
137.9
2.9
139.6
12.1
139.5
10.2
143.2
6.3
139.4
10.0
Overbleached Hair
KP
ELP-KP
BSK
Hydrolized Keratin

Figure 3

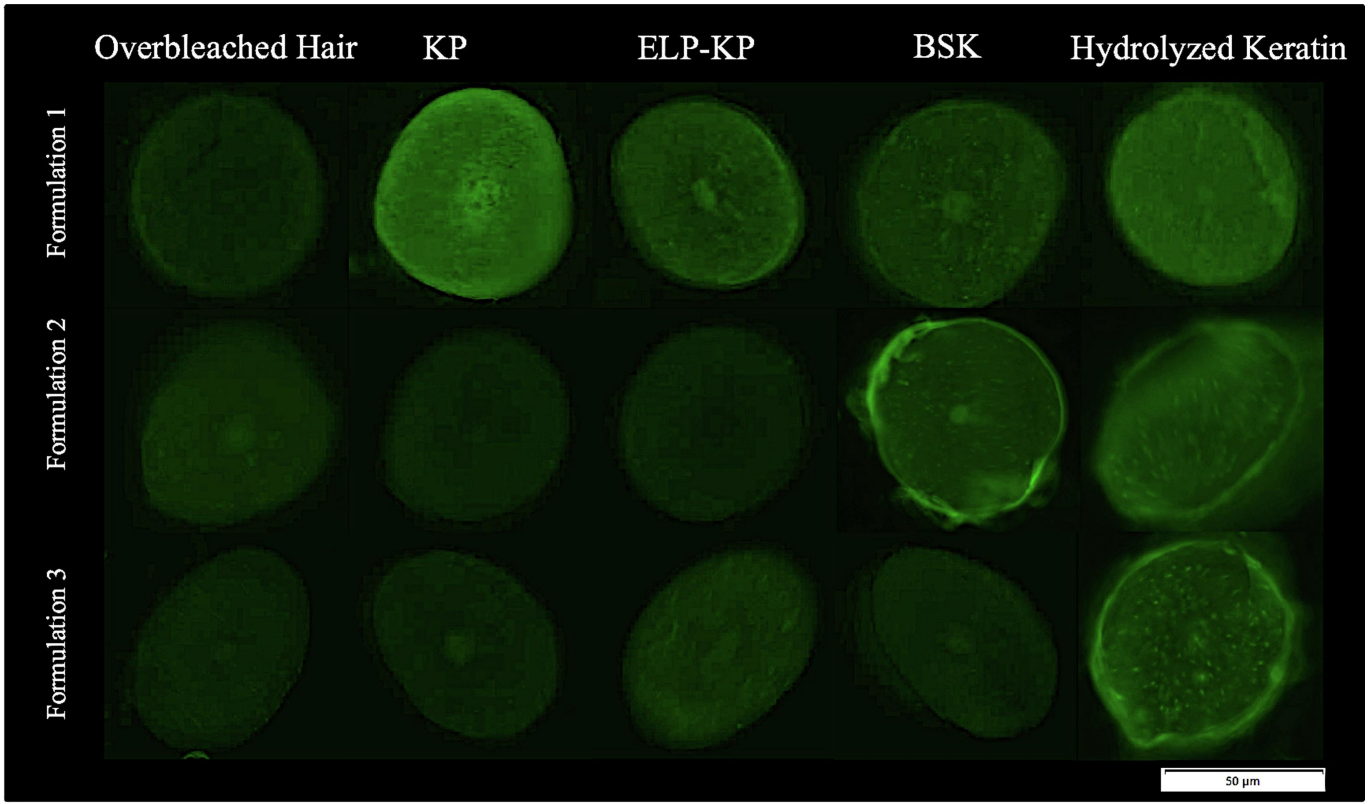

Overbleached Hair
KP
ELP-KP
BSK
Hydrolyzed Keratin
Formulation 1
Formulation 2
Formulation 3
50 µm

Figure 4