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Proteins as Hair Styling Agents

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Abstract: The perming of hair is a common styling procedure with negative impact on the overall properties of the hair fibers. Usually, this process uses harsh chemicals to promote the disruption of disulfide bonds and the formation of new bonds to change the shape of hair. Here, we explored bovine serum albumin (BSA), silk fibroin (SF), keratin and two fusion recombinant proteins (KP-UM and KP-Cryst) as new perming agents. A phosphate buffer prepared at different pH values (5, 7 and 9) was used to apply the proteins to virgin Asian hair, and a hot BaByliss was used to curl the hair fibers. To assess the potential of the protein formulations for hair styling, the perming efficiency and the perming resistance to wash were measured. Furthermore, the fiber water content was evaluated to assess if the proteins protected the hair during the styling process. Despite all of the proteins being able to assist in the curling of Asian hair, the best perming efficiency and perming resistance to wash results were observed for BSA and keratin. These proteins showed perming efficiency values close to that measured for a commercial perming product (chemical method), particularly at pH 5 and 9. The increase in the hair's internal and external water contents revealed a protective effect provided by the proteins during the application of heat in the styling procedure. This study shows the potential of proteins to be used in the development of new eco-friendly hair styling products.

Keywords: hair; proteins; perming efficiency; perming resistance; green formulations

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1. Introduction

Hair has an important psychosocial role throughout life, by influencing individual self-perception and helping in the creation of bonds between individuals. Despite being a physical feature, we can easily change its length, color and shape with a plethora of different hair cosmetic products available on market [1,2]. Structurally, hair has a mesh-like organization composed mainly by keratin, for which the α-helices are coiled by ionic forces, hydrogen bonds, van der Waals forces and disulfide bonds [3]. The weaker bonds can be easily broken and reformed by simply styling wet hair; however, this new shape is only temporary [2]. To permanently change the hair shape such that it persists through several washes with shampoo, it is necessary to reconfigure the disulfide bonds via the reduction and reformation of new disulfide bonds using perming and relaxing solutions [3,4].

The permanent waving is generally a two-step process based on the action of reducing agents, thioglycolates or bisulfites, followed by an oxidative agent, normally hydrogen peroxide [1]. The first step consists in the application of an alkaline reductive solution on the hair to disrupt the disulfide bonds. This alkaline solution, with a pH of around 9, promotes lifting of the hair cuticles and penetration of the solution onto the hair cortex. The perming solution is then washed from the hair, and a neutralizing solution containing an oxidative agent is applied over the hair to promote the formation of new bonds according to the new desired hair shape [2,5].

Despite being a common procedure in hair styling, hair becomes extremely damaged after repeated chemical perming. This process promotes the removal of the hydrophobic

top-layer of the cuticle; the appearance of cracks; the lifting of hair cuticles; the appearance of split ends; and changes in hair texture, hydration and gloss [3].

Besides the negative impact on the quality of the hair fibers, many of the products used during perming contain toxic chemicals, with a negative impact on human life and environment [6–10]. These chemicals may include alkaline agents, thioglycolates, guanidine and even formaldehyde [3]. The increasing consciousness about health and the environment worldwide has promoted the development of green formulations to replace the toxicity of the current perming methods. The development of innovative green hair cosmetic products is foreseen as the best alternative to the products available on the market, which is commonly composed of chemicals.

There are already some nontoxic formulations for hair perming that were developed to substitute the current perming formulations. The alternative proposed by Cruz et al., 2017, is based on the use of keratin decapeptide sequences derived from the human keratin genome. These peptides replace the harsh alkaline reductive solutions, minimizing or even avoiding the damage caused to the hair fibers by chemical products [3]. In the work of Song et al., 2016, the capacities of cysteine and polycarboxylic acids to substitute the thioglycolates and the hydrogen peroxide were demonstrated, respectively. Cysteine, an amino acid with a thiol group and a strong reducibility, reduces the disulfide bonds, while the polycarboxylic acids, known to have the ability to crosslink diverse protein materials, promotes the formation of new disulfide bonds [5].

In this study, we explored the potential of bovine serum albumin (BSA), silk fibroin (SF), keratin and two recombinant fusion proteins (KP-UM and KP-Cryst) to be used on the development of alternative eco-friendly formulations to curl straight, Asian hair. The selected proteins present different structures and properties (Table 1) that might influence the protein's ability to model the hair.

Table 1. Molecular weight, isoelectric point and structure of bovine serum albumin (BSA), silk fibroin, keratin, KP-UM and KP-Cryst proteins.

Protein	Molecular Weight (kDa)	Isoelectric Point	Structure
BSA	66.5	4.7 [11]	Globular
Silk Fibroin	Heavy chain 390 [12] Light chain 26	2.1 [13]	Anti-parallel β-sheet
Keratin	44 to 66 [14]	(4.9–5.4) * (6.5–8.5) ** [15]	α -helix
KP-UM	26.3	8.39	β-barrel
KP-Cryst	24.8	7.13	Greek Key

* Isoelectric point of type I keratin. ** Isoelectric point of type II keratin.

Serum albumin is the most abundant protein in mammal plasma and has several important functions. Particularly, BSA is a multifunctional protein with a remarkable ligand binding capacity, with the capacity to transport a variety of metabolites [16,17]. Silk fibroin (SF) is obtained from silkworm *Bombyx mori* and consists of two chains (H-fibroin (heavy with 390 kDa) and L-fibroin (light with 26 kDa)) connected by a disulfide bond [18]. The SF is considered nontoxic and biocompatible and presents outstanding water binding and absorption abilities, which make this protein ideal for cosmetic applications [19].

Keratin proteins are a broad group of insoluble proteins found in the hair, making up more than 75% of his weight and having 7–20% cystine and a α-helix structure [20,21]. Due to its outstanding mechanical and protective properties, keratin is widely explored for the development of cosmetic formulations. Keratin and primarily keratin hydrolysates have been commonly used in personal and hair care products [22].

The KP-UM is a fusion protein with the ability to color and improve the mechanical properties of hair. This protein was first described by Tinoco et al., 2019, and is constituted by an ultramarine chromogenic protein (UM) and a keratin-based peptide (KP) linked by a glycine-alanine linker $(GA)_5$. The KP-UM protein undergoes a change in color depending on the polarity and pH of the solution where it is resuspended. Independent of the

formulation, the KP-UM protein is able to dye overbleached hair (at the cuticle and cortex level) while improving the mechanical properties of hair [23].

The KP-Cryst fusion protein combines the thermodynamic stability of the γD-crystallin protein present in the human eye lenses, with the ability of the keratin-based peptide (KP) to bind to the hair. The KP-Cryst shows a great ability to bind to the hair fibers, to improve the hair's mechanical properties and to reduce the water loss of the hair fibers after heat styling procedures [24].

In this work, we evaluated the abilities of BSA, SF, keratin, KP-UM and KP-Cryst proteins to change the shape of virgin Asian hair. The capacity of the proteins to modulate the shape of hair was determined in terms of hair perming efficiency across six wash cycles. To validate the proteins as potential products for hair styling, the perming efficiency determined for each protein was compared with the results obtained for a commercial chemical-based method. Moreover, the protective effect of the proteins and the relation between the hair water content after perming and the capacity to retain the curling effect was also investigated.

2. Materials and Methods

Natural Asian, black hair samples were provided by International Hair Importers & Products Inc. A local hairdresser provided the hair samples used for keratin extraction. The KP-UM and KP-Cryst genes were synthesized by GenScript and cloned in the pET-28 a (+) plasmid. Nickel magnetic beads for protein purification were available from GenScript (Piscataway, NJ, USA). The silk fibroin (SF) was extracted from *Bombyx mori* cocoons donated by Dr. Silvia Cappellozza from 'Sezione Specializzata per la Bachicoltura' (Padova, Italy). The BSA protein was obtained from Merck (Madrid, Spain). The culture medium was purchased from Grisp, Portugal, and the detergent compatible DC protein assay kit was obtained from Bio-Rad, Portugal. All of the other chemicals were supplied by Merck (Madrid, Spain).

2.1. Expression and Purification of KP-UM and KP-Cryst Fusion Proteins

The KP-UM and KP-Cryst fusion proteins were expressed and purified as described by Tinoco et al., 2019 [23,24]. Shortly, *Escherichia coli* BL21(DE3) harboring the pET-28 a (+) vector with the KP-UM and KP-Cryst genes was grown in Terrific Broth-Auto Induction Medium (TB-AIM) with kanamycin for 24 h at 37 °C, 200 rpm. The harvested cells were resuspended with lysis buffer (20 mM sodium phosphate, 500 mM NaCl at pH 7.4 supplemented with protease inhibitor) and were lysed by sonication (40% amplitude, 3.0 s on plus 9.0 s off for a total of 10 min on) in a sonicator vibracell^{TM} SONICS. The soluble fraction containing the proteins was purified using nickel magnetic beads with specificity to the His-tag sequence present in the N-terminal of both proteins [23,24]. The KP-UM and KP-Cryst fusion proteins were then dialyzed at 4 °C for 3 days against distilled water using a dialysis membrane with a 14 kDa cutoff.

2.2. Keratin Extraction and Purification

Keratin proteins were extracted from donated human hair provided by a local hairdresser. After the contaminants and lipids were removed from hair according to the IAEA/RL/50 1978 recommendations, the keratin was extracted using a protocol adapted from Ayutthaya et al., 2015 [25]. The hair was mixed with a solution containing 8 M urea, 0.2 M SDS and 0.5 M of sodium metabisulphite in a ratio of 10:1 (mL of solution to grams of dry hair). The mixture was heated at 100 °C for 30 min and then incubated overnight at 37 °C with constant agitation. The extraction solution was centrifuged at 2800 *g* for 10 min, and the supernatant was filtered to remove hair fragments. The keratin solution was then dialyzed at room temperature for 5 days against distilled water using a dialysis membrane with a 14 kDa cutoff [22].

2.3. Silk Fibroin (SF) Extraction and Purification

The *Bombyx mori* cocoons (5 g) were immersed in 1 L of boiling 0.05 M Na_2CO_3 solution for 10 min. The Na_2CO_3 solution was changed several times until complete fragmentation of the cocoons. The degummed silk fibroin (SF) was dried at 40 °C and solubilized with a solution of 9.3 M lithium bromide at 60 °C. The soluble SF was filtered to remove undissolved fibers and dialyzed for 5 days in distilled water using a dialysis membrane with a 14 kDa cutoff [26].

2.4. Hair Perming with Keratin, KP-UM, SF, BSA and KP-Cryst Proteins

The Asian virgin hair was pretreated in order to increase the proteins' binding to the hair, prior protein application and hair styling. Initially, hair meshes with the same length and weight (150 mg) were incubated with 2 mL of a 2 M urea solution, pH 9.5, for 20 min and then were washed and dried with a hairdryer. Afterwards, the hair meshes were incubated with 2 mL of an ethanolic formulation (0.1 M phosphate buffer (pH 5, 7 or 9)) with 1.5% (*v*/*v*) propylene glycol, 0.5% (*v*/*v*) benzyl alcohol and 10% (*v*/*v*) ethanol) for 20 min. The hair was dried with a towel, and 2 mL of the protein solutions (1 mg/mL in phosphate buffer pH 5, 7 or 9) was applied over the hair for 20 min. The control samples (ethanolic formulation) were incubated after pretreatment with phosphate buffer prepared at pH 5, 7 and 9 without the proteins. A commercial BaByliss previously heated at 200 °C, was used for 50 s to curl the treated hair meshes. This time was considered the time necessary for the hair to dry and to acquire a curly shape. To compare the initial results, all of the hair meshes were curled the same number of times and in the same direction around the BaByliss.

2.5. Perming Efficiency

The perming efficiency of the curled Asian hair was evaluated after BaByliss application and across six wash cycles, with tap water and a commercial shampoo (Pantene® Basic). The wash cycles were performed without adjusting the pH of the wash solution (pH 6.5). After washing, the meshes were air-dried at room temperature and the perming efficiency was determined when the meshes were completely dried (Figure 1). For comparison, a commercial perming solution (FarmaVita® Life the Perm) (water, ammonium thioglycolate, ammonium bicarbonate, ethanolamine, PEG-40 hydrogenated castor oil, cocamidopropyl betaine, ammonium hydroxide, polyquaternium-7, simethicone, styrene/vp copolymer and tetrasodium EDTA) and a neutralizing solution (FarmaVita® Universal Neutralizer) (water, SDS, hydrogen peroxide, simethicone, phosphoric acid and oxyquinoline sulfate) was selected as a control. The perming efficiency was calculated using the following equation [27]:

$$\text{Perming efficiency } (\%) = \frac{\frac{\text{number of loops after perm}}{\text{fiber length after perm}}}{\frac{\text{number of loops before perm}}{\text{fiber length before perm}}} \times 100 \quad (1)$$

2.6. Perming Resistance to Shampoo

In order to determinate the resistance to washing of the protein's treatment, the perming resistance was calculated after six wash cycles using the following equation:

$$\text{Perming resistance } (\%) = \frac{\text{Perming efficiency}_{after\ washing}}{\text{Perming efficiency}_{\text{before washing}}} \times 100 \quad (2)$$

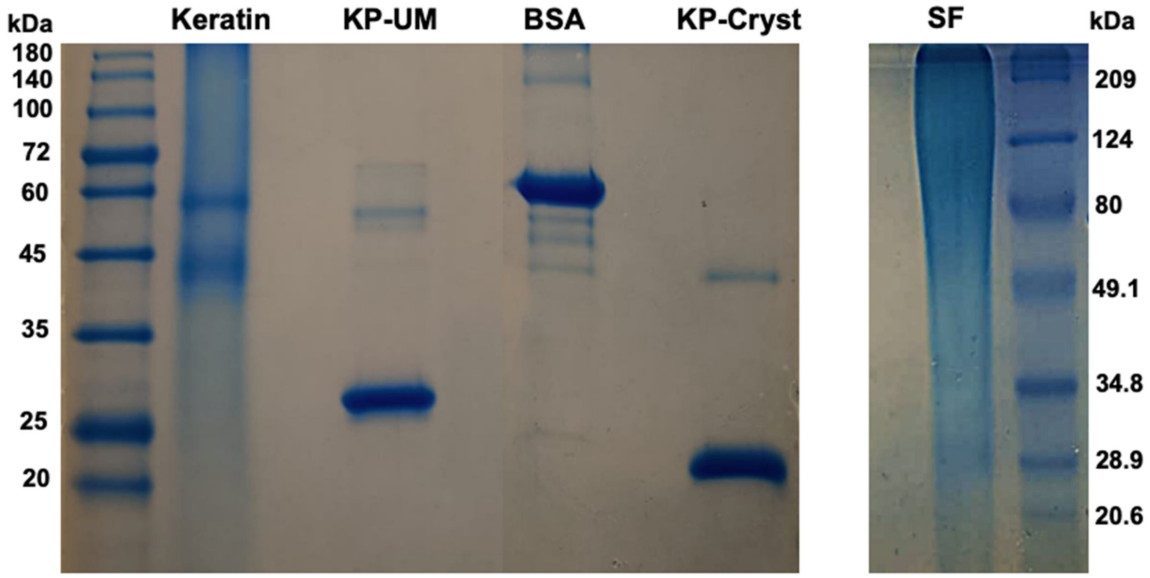

Figure 1. Protein purity analysis by SDS-PAGE in a 12.5% polyacrylamide gel. The tested proteins included keratin, KP-UM, BSA, KP-Cryst and silk fibroin (SF) proteins.

2.7. Hair Moisture Content

To determine the moisture content of the hair with and without treatment, a thermal gravimetric analysis (TGA) instrument (Perkin Elmer, Waltham, MA, US) was employed. The hair samples were transferred to an alumina crucible until they reached a weight between 8 and 10 mg. The temperature calibration was performed using Curie temperatures of the reference materials: alumel, nickel and perkalloy at the same sample scanning rate. The measurements were all performed under a nitrogen atmosphere (flow rate: 20 mL/min), heated from 25 to 65 °C at 20 °C/min and maintained for 40 min. Thereafter, the temperature was increased from 65 to 180 °C at 20 °C/min, which was maintained for 30 min to evaporate all of the water contained in the hair. All of the measurements were performed in duplicate, and the mean values and standard deviations for external, internal and total water content were determined [28].

2.8. Statistical Analysis

The data are presented as average standard deviation (SD), n = 3. Statistical comparisons were performed by one-way ANOVA with GraphPad Prism 5.0 software (La Jolla, CA, USA, 2016 r). Dunnett's test was used to compare the results with a specific control. A p-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Perming efficiency

In this study, we evaluated the ability of different proteins (keratin, KP-UM, silk fibroin, BSA and KP-Cryst) to curl straight Asian hair. To maximize the binding of the proteins to the hair fiber, their penetration into the hair cortex and the curling effect, the proteins were incorporated into a phosphate buffer with different pH values (5, 7 and 9). To promote the swelling of the hair fibers and the lifting of the cuticles [29,30], the hair tresses were pretreated with an urea solution followed by the ethanolic formulation prepared at pH 5, 7 and 9 prior to treatment with the proteins. To curl and fix the new shape, the hair tresses were thermally treated using a hot BaByliss for 50 s.

With the goal of understanding if and how the proteins' conformation structure influence the protein capacity to curl Asian hair, we selected five proteins with different structural conformations: ß-sheets (silk fibroin) [31], α-helix (keratin) [15], globular (BSA) [32], ß-barrel (KP-UM) [23] and Greek-key motifs (KP-Cryst) [24]. Protein's purity was assessed

by SDS-PAGE (Figure 1) prior to application to the hair. Analyzing the gel, high purity can be observed for the KP-UM and KP-Cryst fusion proteins. The higher molecular weight bands detected for these two proteins correspond to the respective dimmers. The presence of two cysteine residues on the KP sequence might promote the formation of these structures. The BSA presents an intense band with the expected size (66.5 kDa); however, this protein has a lower degree of purity since other lower molecular weight bands can also be observed. Both keratin and silk fibroin present diffuse patterns through the gel [18,21], with more intense bands around 43 and 58 kDa for the keratin and around 29 kDa and higher than 209 kDa for the silk fibroin.

After perming, the hair shape was analyzed relative to the number of loops and hair length (Figure 2) for the different proteins before and after each wash cycle (six cycles in total).

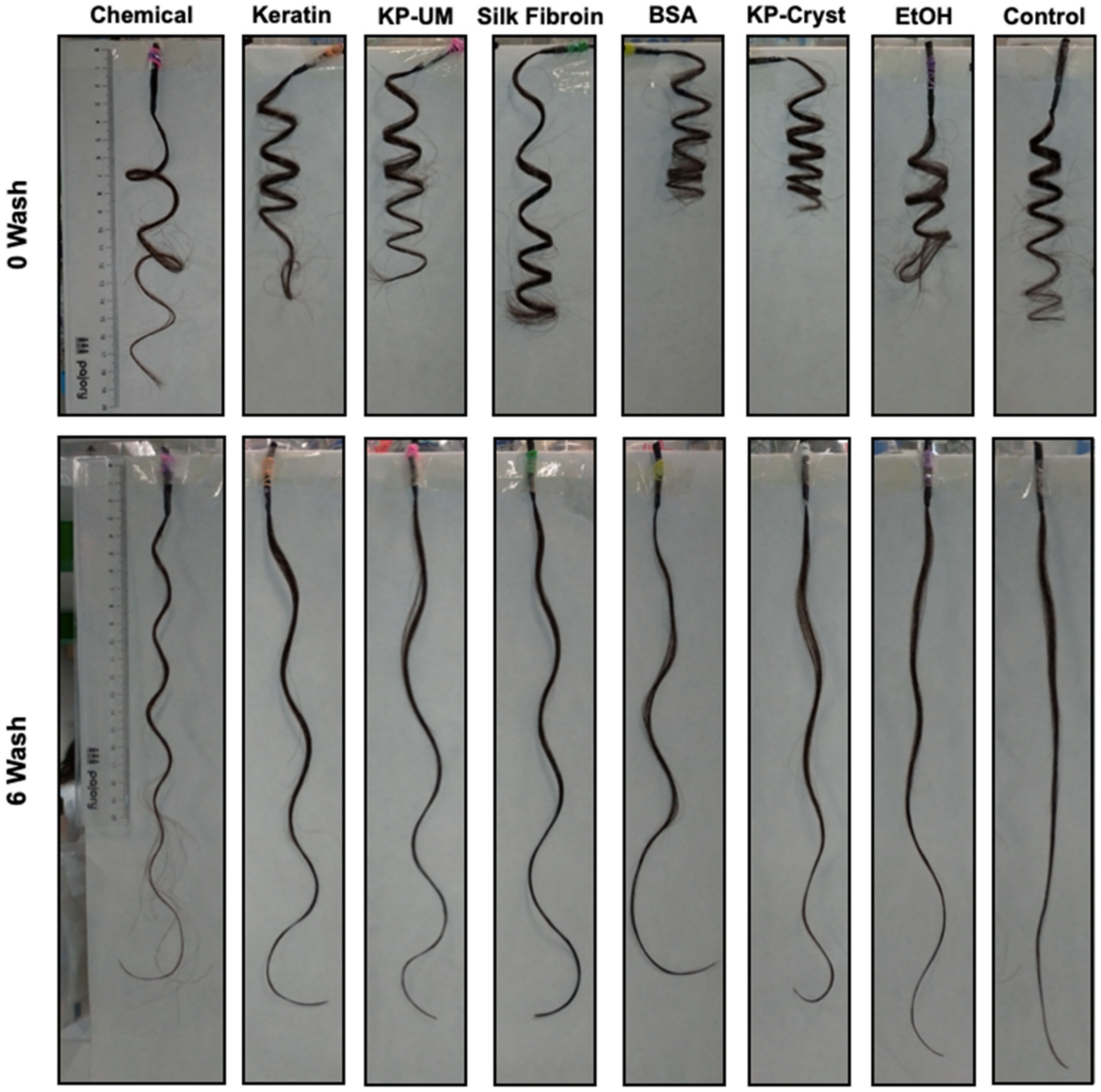

Figure 2. Effect of hair washing with a commercial shampoo on the curliness of Asian hair. The hair tresses were treated with keratin, KP-UM, silk fibroin, bovine serum albumin (BSA) and KP-Cryst proteins dissolved in phosphate buffer (pH7) before curling using a hot BaByliss. Hair treated with a commercial formulation for perming ("Chemical"), treated with the ethanolic formulation and phosphate buffers ("EtOH"), or without any treatment ("Control") were used as controls for the proteins' capacity to curl the Asian hair.

The number of loops and the hair length were used to calculate the perming efficiency as a measure of the capacity of the keratin, silk fibroin, BSA, KP-UM and KP-Cryst proteins to curl hair and to maintain hair shape throughout wash cycles [27].

The perming efficiency of the hair treated with the different proteins was compared with the perming efficiency of the Asian hair without treatment (Control) (Figure 3) and with the perming efficiency of the hair treated with the respective ethanolic formulation/phosphate buffer (EtOH formulation) (Figure 4). The results presented on both figures were determined using the number of loops and the fiber length after six wash cycles with shampoo.

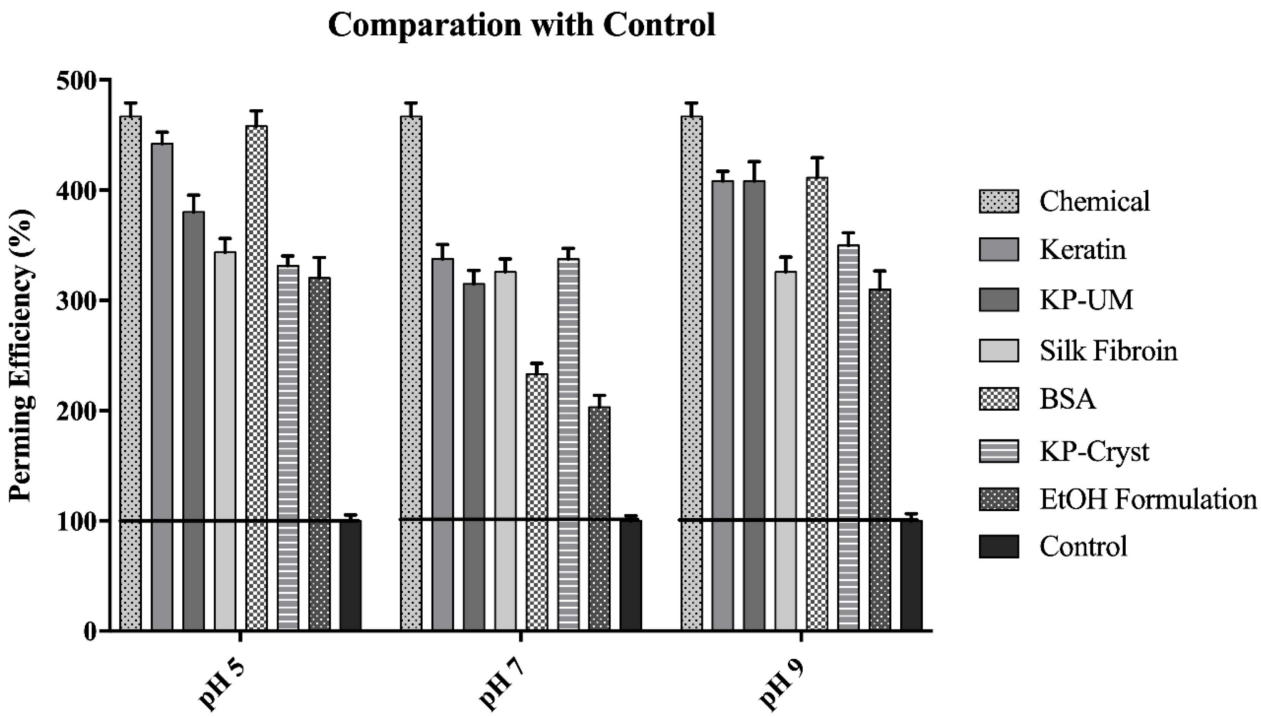

Figure 3. Perming efficiency of the hair tresses treated with the keratin, KP-UM, silk fibroin, BSA and KP-Cryst proteins prepared in phosphate buffer at pH 5, pH 7 or pH 9, compared to the "Control" samples. Hair treated with a commercial formulation for perming ("Chemical"), treated with the ethanolic formulations and phosphate buffers ("EtOH formulation"), or without any treatment ("Control") were used as controls for the proteins' capacity to curl Asian hair. The values represent the mean ± SD of two measurements. The data were analyzed by Dunnett's multiple comparison test, and all of the samples show significative differences (p-value ≤ 0.0001) when compared with the "Control" data.

Analyzing Figure 3, it was observed that all of the tested conditions presented significantly higher perming efficiencies (p-value ≤ 0.0001) than the control sample (no treatment) after six wash cycles. As expected, the best perming efficiency was found for the chemical treatment. However, perming efficiencies similar to the chemical treatment were obtained for BSA and keratin at pH 5 and 9. When compared with the perming efficiency of the chemical method, decreases of 2% (pH 5) and 12% (pH 9) were observed for the hair treated with BSA and decreases of 5% (pH 5) and 13.5% (pH 9) were observed for keratin. A good perming efficiency was also observed for the hair treated with the KP-UM protein at pH 9.

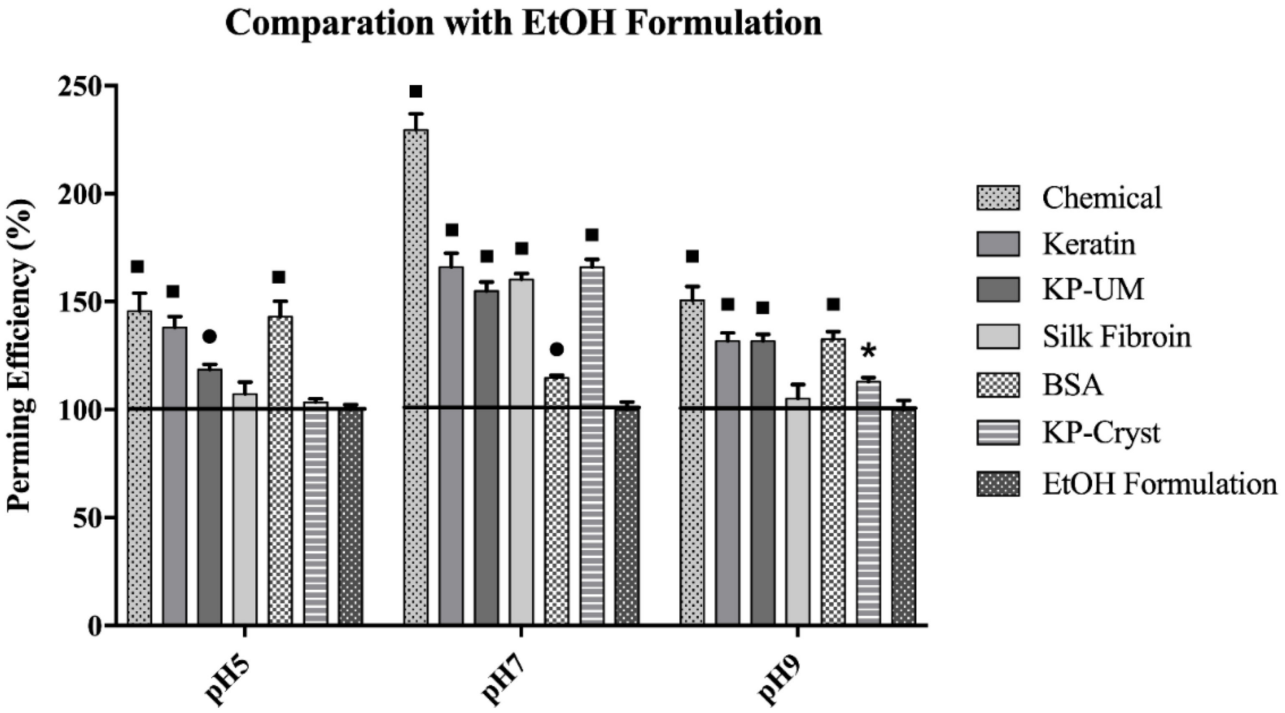

Figure 4. Perming efficiency of the hair treated with the keratin, KP-UM, silk fibroin, BSA and KP-Cryst proteins prepared in phosphate buffer at pH 5, pH 7 or pH 9 compared to the respective "EtOH formulation" sample. Hair treated with a commercial formulation for perming ("Chemical"), treated with the ethanolic formulation and phosphate buffers ("EtOH Formulation"), or without any treatment ("Control") were used as controls for the proteins' capacity to curl Asian hair. The values represent the mean ± SD of two measurements. The data were analyzed by Dunnett's multiple comparison test: p-value ≤ 0.05 (*), p-value ≤ 0.01 (•), and p-value ≤ 0.0001 (■) compared to the respective "EtOH formulation" data.

Despite the good results obtained for keratin, KP-UM and BSA proteins at pH 5 and 9, lower perming efficiencies were observed for the samples tested at pH 7. However, these seem to be related to the effect of pH on the hair fibers. Comparing the results of the hair treated with the ethanolic formulations at pH 5 and 9 with the results of the hair treated with the ethanolic formulation at pH 7, decreases of about 34% and 37%, respectively, for this sample was observed.

To determine the isolated effect of each protein, the perming efficiency of the hair treated with the different proteins was compared with the perming efficiency of the hair treated with the respective ethanolic formulation (EtOH formulation) (Figure 4).

Comparing the perming results of the hair treated with proteins with the respective ethanolic formulations, higher perming efficiencies were obtained for the hair fibers treated with the proteins' in the ethanolic formulation prepared at pH 7. The higher contribution of the proteins at this pH could be explained by the low perming efficiency obtained for the hair treated only with the ethanolic formulation at pH 7. Solutions with pH values close to 7 seem to influence the ratio between the number of salt bridges and hydrogen bonds, which are described to be involved in the shape of the hair fibers [33]. In this way, the low perming efficiency observed for the control at pH 7 highlights the effect of the proteins in the shaping of the hair fibers. An exception was observed for BSA, where the lowest perming efficiency (14.81%) was obtained for this pH value. This behavior could be explained by a more compact conformation of BSA at neutral pH [34]. As we move to other pH values, there is a relaxation in the BSA's structure, which may favor the interaction of this protein with hair fibers, increasing the perming efficiency at pH 5 and pH 9 [34].

The goal of this work was to explore different proteins as valid alternatives to the chemical treatment usually applied to change the shape of hair. To analyze if the tested proteins show potential as hair perming agents, the perming efficiencies of the hairs treated with the proteins were compared with the perming efficiency of the chemical method.

In Table 2, the values for the perming efficiency ratios determined between the proteins treated hairs and the chemical treated hair are presented.

Table 2. Perming efficiency ratios of the hair treated with keratin, KP-UM, silk fibroin, BSA and KP-Cryst proteins prepared in phosphate buffer at different pH values. The ratios were calculated between the perming efficiency results of the hair treated with the protein formulations and the hair chemically treated.

	Perming Efficiency Ratio (Sample/Chemical)		
Sample	**pH 5**	**pH 7**	**pH 9**
Keratin	0.947	0.723	0.875
KP-UM	0.815	0.675	0.872
Silk Fibroin	0.736	0.698	0.698
BSA	0.982	0.500	0.882
KP-Cryst	0.711	0.723	0.750
EtOH Formulation	0.686	0.435	0.664

The perming efficiency ratio provides a measure of a protein potential to be used as a perming agent when compared with the common chemical methods. Perming efficiency ratios close to one indicate that the protein has a perming capacity very similar to that of the chemical-based method.

According to the results present in Table 2, we organized the protein formulations in ascending order considering their capacity to curl Asian hair:

pH 5 EtOH formulation < KP-Cryst < Silk fibroin < KP-UM < Keratin < BSA
pH 7 EtOH formulation < BSA < KP-UM < Silk fibroin < KP-Cryst < Keratin
pH 9 EtOH formulation < KP-Cryst < Silk fibroin < KP-UM < Keratin < BSA

While BSA presented the highest potential to curl Asian hair at pH 5 and 9, but not at pH 7, the keratin showed good perming performances for all of the tested pH values, with perming efficiencies between 72% and 95% when compared with the chemical method. These results could be related to the fact that the tested keratin was extracted from human hair samples, thus presenting a high sequence identity with the hair, which could improve the keratin binding and perming performances [35]. Moreover, the high cysteine content of keratin could favor the formation of disulfide bonds with the high-sulfur hair matrix, improving the perming efficiency results [36].

Considering the protein's secondary structure, better perming results were obtained for proteins with a high α-helix content, such the keratin and the BSA proteins. Keratin presents 38–45% of the protein structure in its α-helical conformation [15], while BSA presents a total content of 67% [37]. On the other hand, the proteins with the highest ß-sheet contents (KP-UM, KP-Cryst and silk fibroin) presented lower perming capacities when compared with the chemical method. Despite the apparent contribution of the proteins' structure to their capacity to change the shape of hair, molecular dynamics simulations (MD) with pulling/stretching assays help to understand the mechanisms behind the effect of a protein structure on its ability to bind and to change the shape of hair. These MD studies will be subject of a future publication.

An effect of protein size could also be hypothesized to influence the proteins' potential to change hair shape. The best results were obtained for proteins with an intermediate molecular weight between 44 and 67 kDa, particularly keratin and BSA (Table 1). Regarding silk fibroin, this protein has the highest molecular weight (416 kDa) and the lowest perming efficiency. Most likely, due to its size, SF is located mainly at the surface of the hair fiber. Although the KP-UM and KP-Cryst proteins, with molecular sizes around 24–26 kDa, might penetrate deeper into the fiber, their smaller size could diminish the number of possible interactions with the proteins from the hair, affecting their ability to style hair [38].

Generally, it seems that the capacity of each protein to shape hair is related to the proteins' amino acidic sequence, its conformation and size, the charge at the proteins' surface and their capacity to interact with the proteins from hair.

The results obtained for the proteins' perming efficiencies are corroborated by the visual inspection presented in Figure 2. All of the protein's formulations improved the curliness of hair relatively to the respective ethanolic formulation. Moreover, some of the proteins presented perming results similar to that obtained for the chemical method.

3.2. Perming Resistance to Shampoo

The perming resistance to shampoo was determined by comparing the perming efficiency after six wash cycles with the perming efficiency before any washing step (Table 3). To determine the perming resistance to shampoo, we mimicked the conditions normally used in a daily routine: using tap water and a commercial shampoo brand (Pantene®Basic), without any pH adjustment. The perming resistances compared in all of the wash cycles with the perming efficiency before any washing step are represented in Figure 5. This parameter provides information about how strong is the protein effect during hair curling. Hair with high perming resistances to shampoo maintained the desired hair shape longer, while hair with low perming resistances lose the curled shape within the first few wash cycles. It is important to note that a higher perming resistance to washing does not reflect higher perming efficiency results.

Table 3. Hair perming resistance to washing. The resistance values were obtained by comparing the perming efficiencies after six wash cycles with the perming efficiencies before any wash step.

	Perming Resistance (%)		
	pH 5	**pH 7**	**pH 9**
Chemical	70.37	70.37	70.37
Keratin	48.65	29.66	47.64
KP-UM	28.97	24.00	37.07
Silk Fibroin	41.45	30.00	36.08
BSA	93.58	11.85	35.26
KP-Cryst	36.49	17.36	40.83
EtOH Formulation	33.90	15.32	29.29
Control	7.62	7.62	7.62

Comparing the results obtained for the loss of perms after six wash cycles, the hair treated with BSA in ethanolic formulation at pH 5 presented the highest perming resistance to shampoo. The effect of BSA on the hair resistance to wash (93.58%) was even higher than that measured for the chemical method (70.37%), pointing out the potential of this protein to be used as a perming agent. For the perming efficiency results, the hairs treated with keratin presented promising results for all of the tested pH, with perming resistance to shampoo ranging from ≈30% for pH 7 to ≈48% for pH 5. The hair treated with the other proteins also presented good perming resistance results; however, the perming efficiency of the hair treated with these proteins was not as high as that for BSA and keratin.

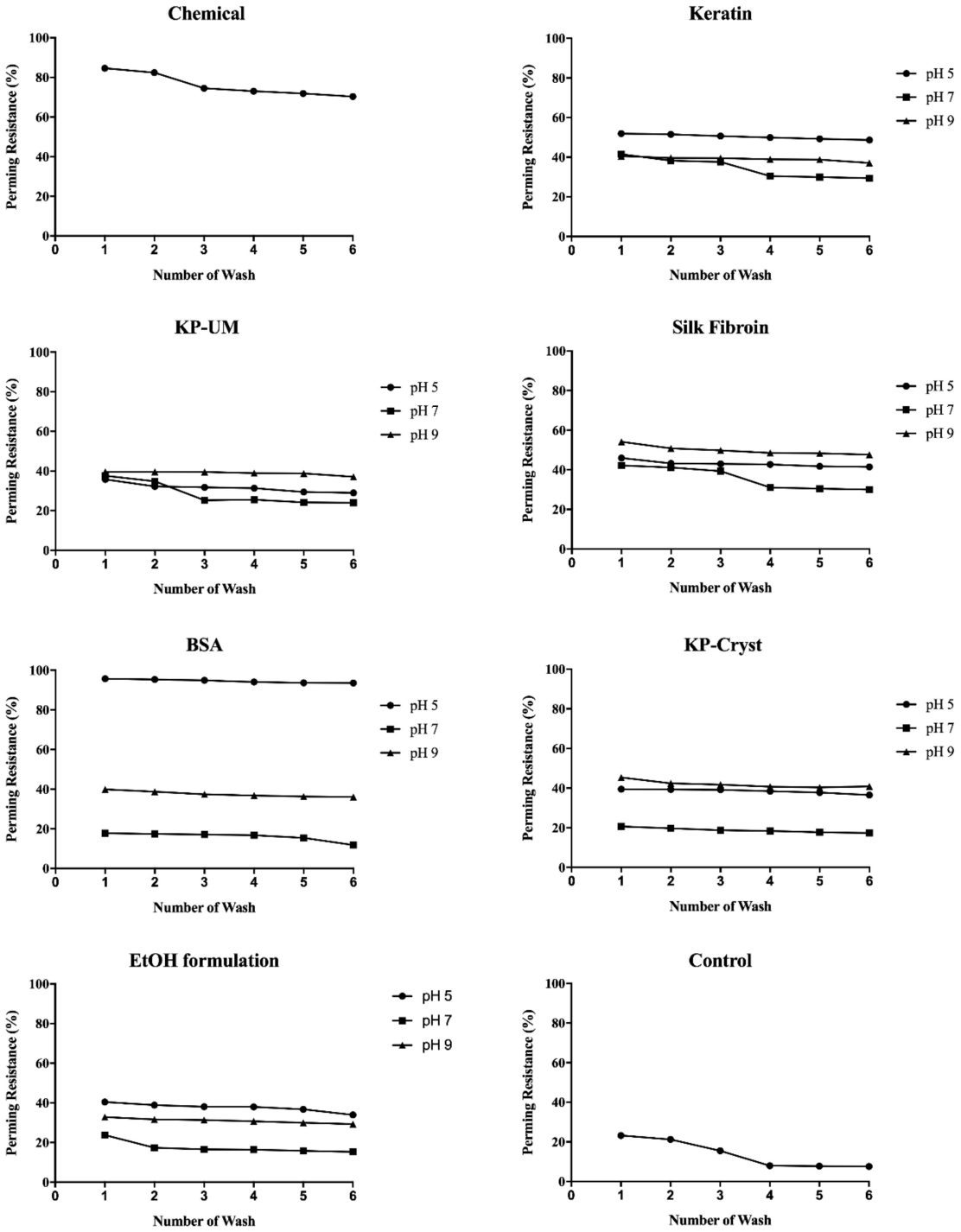

Figure 5. Hair perming resistance across six wash cycles with commercial shampoo. The resistance values were obtained by comparing the perming efficiencies after a certain wash cycle with the perming efficiencies before any washing step.

3.3. Effect of Protein Application on the Fibre Water Content

Water molecules play a vital role in keratinized tissues such as human hair by increasing the fibers' moisture, which affects hair shine, conditioning, manageability and physical properties [39,40]. The water content and absorption profiles could be drastically altered by daily routines or cosmetic procedures that affect the keratin structure of hair [41,42]. Despite water influencing several properties of hair, its effect is more notorious on the mechanical behavior of hair fibers [43]. The capacity of proteins to bind water is related to the proteins' composition and conformation. The interaction between the water

molecules and the polar hydrophilic groups of proteins occurs via hydrogen bonding, with the proteins' capacity to retain moisture being dependent on the type and number of these polar groups [44].

For this study, the water content in the hair fibers was expressed in terms of the percentage of water mass relative to the total hair fiber weight. This parameter was evaluated by thermogravimetric analysis with two heating steps to evaluate the fibers' loss of internal and external water [28].

As observed in Figure 6, there are significant differences (p-value ≤ 0.0001) when comparing the results of the hair samples treated with the proteins and the "Control" sample relative to the internal and external water content. The "Control" sample corresponds to untreated Asian hair, where the BaByliss was applied for a total of 50 s. Considering external water, it was observed that all of the protein formulations were able to significantly increase (p-value ≤ 0.0001) the fibers' external water content, with better results obtained when the proteins were applied in the ethanolic formulation prepared at pH 9. The increase in the fibers' external water content could be justified by the proteins' ability to interact and bind water molecules via hydrogen bonding [44,45].

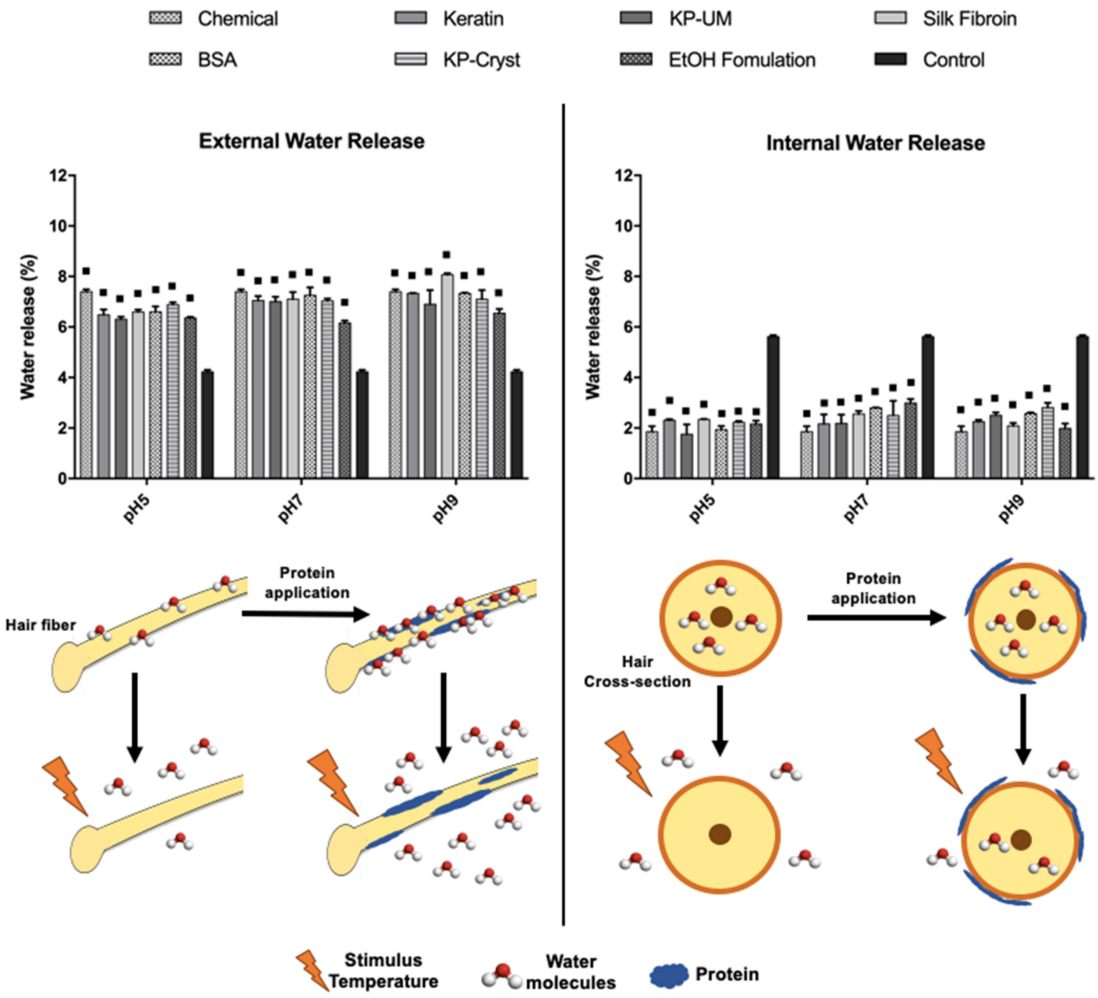

Figure 6. Percentage of external and internal water contents in the hair fibers after treatment with keratin, KP-UM, silk fibroin, BSA and KP-Cryst proteins prepared in phosphate buffer (pH 5, 7 and 9) and BaByliss application for 50 s. Hair treated with a commercial formulation for perming ("Chemical") and hair curled with the BaByliss after treatment with the ethanolic formulation and phosphate buffers ("EtOH formulation"), or without any treatment ("Control") were used as controls. The values represent the mean ± SD of two measurements. The data were analyzed by Dunnett's multiple comparison test: p-value ≤ 0.0001 (■) compared to the "Control" data.

Considering the internal water content, it was observed that the 'Control' sample lost around 2–3 times more internal water content than the other samples. These results could be due to the formation of a protein-based film around the hair fibers that protect the hair against the negative effects of the high temperature procedure. This behavior was already observed for KP-Cryst protein that formed a film-like structure over Asian and Caucasian hair and protected the hair fibers against the high temperature of straightening iron devices [24].

Significative differences (p-value ≤ 0.0001) for internal and external water contents were also verified when compared to hair treated with the ethanolic formulations without the proteins and with the 'Control' sample, demonstrating that the ethanolic formulation used to resuspend the proteins also play an important role in the fibers' water content after BaByliss application. The results obtained for hair permed using the chemical method also demonstrate a decrease and an increase in the internal and external water contents, respectively, when compared with the "Control" data. This behavior could be due to impairment of the keratin structure during the chemical treatment that reorganizes the disulfide bonds of the fiber and increases the porosity of hair [42,46]. The resulting damage causes a disruption of the hair's cuticular sheath, leading to an increase in the fibers' retention of water as well as an increase in the monolayer water absorption capacity [42,46].

4. Conclusions

Hair styling procedures use aggressive chemicals that affect human health and the environment. In this study, we explored BSA, silk fibroin, keratin, KP-UM and KP-Cryst proteins as new perming agents. The perming efficiency of hair treated with these proteins were compared with the results of a commercial product (chemical treatment).

Regardless of the formulation pH, all of the proteins were able to change the shape of hair. The ability of the proteins to style hair, measured in terms of perming efficiency, was dependent on the proteins' amino acidic sequence, size, conformation, charge, and ability to bind to the hair fiber and to the hair proteins. Despite the best perming efficiency being observed for the chemical treatment, keratin and BSA also presented great abilities to change the shape of hair, with perming efficiencies close to that observed for the chemical method.

Moreover, all of the proteins significantly increased the fiber's external and internal water content as a result of the interaction and binding of the proteins to water molecules. The increases in the water content can protect the hair from damage during hair styling procedures using high temperatures.

This study provides new insights about the capacity of proteins to be used as new hair styling agents. The excellent perming efficiencies obtained for the proteins and the high perming resistances to shampoo support their inclusion into more natural and eco-friendly hair cosmetic formulations. Moreover, the great ability of proteins to change the shape of hair point to their potential in replacing the chemicals currently used in hair styling procedures.

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