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Protein disulphide isomerase-mediated grafting of cysteine-containing peptides onto over-bleached hair

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*Department of Textile Engineering, University of Minho, Azurém Campus, Guimarães, Portugal***Abstract**

The ability of Protein disulphide isomerase (PDI) to promote the grafting of two cysteine-containing peptides onto hair was investigated in order to develop an alternative treatment for over-bleached hair. The studied peptides were designed based on human keratin and human lung surfactant proteins and were linked to a fluorescent dye to facilitate visualisation of the grafting process and to assess hair penetration. The ability of the peptides to restore mechanical and thermal properties lost by repeated bleaching treatments was also studied. After eight bleaching treatments, hair samples displayed 42% less mechanical resistance, coupled with a decrease in α -helix denaturation enthalpies and temperatures. Hair surface damage following bleaching was visualized by scanning electron microscopy. Addition of PDI to the treatment formulations promoted peptide attachment to the hair via disulphide bonds, facilitating their penetration into the hair cortex, as observed by fluorescence microscopy. The proposed peptide treatment resulted in an increase in α -helix denaturation enthalpy in over-bleached hair, as well as an increase in both Young's modulus and tensile strength. Thus, mechanical and thermal properties were improved after the peptide treatment in the presence of PDI; suggesting that the formulations presented in this work are promising candidates for hair-care applications.

Keywords: Protein disulfide isomerase, over-bleached hair, cysteine-containing peptides, disulfide bonds, hair treatments

Introduction

Both animal (wool) and human (hair) keratins are characterized by the presence of a broad range of functional groups, such as amines, amides, carboxyls, carboxyamides, hydroxyls and sulphhydryls (Plowman 2007; Block et al. 1939), which are scattered across the two main morphological components of keratins: the fibrous cortex and the cuticle surface (Wolfram 2003; Franbourg et al. 2003). This chemical versatility makes keratin fibres suitable substrates for enzymatic modification, which is considered to be safer than conventional chemical modification. In the case of wool, enzymatic functionalisation is usually performed directly on the wool surface in order to overcome undesired effects such as shrinkage and felting, which can be provoked by the presence of cuticle scales (Negri et al. 1993; Araújo et al. 2009), or to improve its mechanical properties (Cortez et al. 2004, 2005). Similarly,

enzyme functionalisation has also been employed in hair treatments, such as shaping or dyeing. For example, transglutaminases have been used to covalently graft amine-containing compounds to glutamine residues in keratins from skin, hair or nails (Richardson et al. 1996). In addition, gentle but permanent hair relaxation and straightening has been achieved by using a keratinase (kerA), a serine protease isolated from *Bacillus licheniformis* which cleaves inter-peptide bonds allowing the hair fibre to be relaxed and straightened with less damage than traditional straightening methods (Presti 2010).

However, although one of the most interesting characteristics of keratinous fibres is their high cysteine disulfide bond content (Bradbury 1973), this functional moiety has not been extensively explored for enzymatic modifications. Disulphide bonds are the main covalent crosslinks present in keratins, and impart good mechanical and thermal

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properties, through the formation of a three-dimensional structural network (Franbourg et al. 2003; Danciulescu et al. 2004; Feughelman 1997; Plowman 2003).

PDI (EC 5.3.4.1) is a multifunctional enzyme, which is able to catalyse thiol-disulphide exchange reactions in cysteine-containing proteins (Wilkinson & Gilbert 2004), thus promoting oxidative protein folding and the rearrangement of non-native disulphide bonds in proteins (Appenzeller-Herzog & Ellgaard 2008). In addition, PDI-catalysed thiol-disulphide exchange reactions can be controlled by regulating the state of the enzyme active site (Fernandes et al. 2011). Because of these properties, PDI is ideal for grafting functional molecules to hair through disulphide bond formation.

Several studies have reported the use of PDI for the treatment of human and animal hair fibres, by targeting disulfide bonds. For example, King and co-workers demonstrated that PDI is able to partially restore the original properties of aged or harshly treated wool (King & Brockway 1992). PDI was also used by Brockway (1992) to catalyse curling, waving or straightening treatments under mild conditions. For the same purpose, a thioredoxin-like protein (a fragment of PDI) was used to catalyse the treatment of hair with small amounts of thioglycolic acid, a commonly used hair relaxation agent (Pigiet 1990).

The aim of the present study was to assess the effect of the application of two specially designed (engineered) peptides, derived from human lung protein surfactant B (SPB) and human hair keratin (KP), on the strength and integrity of over-bleached hair. PDI was added to the hair formulations to stimulate peptide penetration into the hair cortex, and to catalyse peptide grafting onto the hair fibres. Mechanical resistance, denaturation temperature and enthalpies of the treated hair were analyzed.

Materials and methods

Materials

Stock solutions of bovine liver PDI (Sigma) were prepared in phosphate buffer pH 7.5 at a protein concentration of 0.05 g/L and 17 U/mL. All other chemicals were of analytical grade. Natural European blond human hair samples were provided by International Hair Importers & Products Inc. (New York).

Engineered peptides. The peptides used in this study were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany), and were designed based on: (1) a fragment of keratin intermediate filament protein (KP); and (2) a fragment of mammalian surfactant pulmonary B protein (SPB). Peptides were covalently

Table I. Peptide nomenclature and characteristics: sequence, number of amino acids, molecular weight, isoelectric point, and zeta potential. All peptides were labelled with a fluorescent dye (TAMRA) at their N terminal amines.

Peptide name	Peptide Sequence N-C	N° aa	Mw (Da)	Isoelectric point (pI) ^a	Zeta potential at pH 7.5 (mV)
KP	X ₃ CX ₃ CX ₃	13	1599.84	5.2	4.28 ± 1.21
SPB	XCX ₇	9	1496.87	11.3	-24.93 ± 0.71

^aCalculated with 'Peptide Property Calculator', which is available at www.innovagen.se.

linked via their N-terminal amines to a fluorescent dye (5(6)-carboxytetramethyl-rhodaminesuccinimidyl ester, 5(6)-TAMRA; with $\lambda_{\text{ex}} = 544$ nm and $\lambda_{\text{em}} = 572$ nm), to facilitate analysis of peptide penetration.

To prepare the hair treatment formulations, peptides were dissolved in 0.05 M phosphate buffer (PB) at pH 7.5 and a concentration of 0.6 mM. Characteristics of the peptides are shown in Table I. Peptide sequences are shown in single-letter code, where X is any amino acid residue other than cysteine which is represented by the letter C.

Methods

Hair treatments. Blond hair fibre samples (0.1 g) were washed before treatment with a commercial shampoo. Two different types of hair were treated: (1) virgin hair (VG), defined as hair without any chemical treatment; and (2) hair subjected to eight cycles of bleaching (8 × B). Bleaching consisted of the application of 12% H₂O₂ (v/v) in the presence of 0.1 M Na₂CO₃/NaHCO₃, pH 9.0 buffer at 50°C for 1 h, in a bath ratio of 1:10. Procedures were applied to the same tresses of hair. Hair samples were then subjected to peptide treatment. For peptide treatments, 100 µL of 0.05 mg/mL PDI in 0.05 M PB, pH 7.5, was added to 600 µL of the peptide treatment formulation, and the resulting solution was diluted to 3.0 mL using the same buffer. Final peptide and PDI concentrations were 120 µM and 1.7 µg/mL, respectively. Peptide treatments were performed for 1 h at 37°C. For comparison, hair samples were treated in parallel with the same peptide formulations in the absence of PDI. Subsequently, all samples were thoroughly washed in tap water with a commercial shampoo.

Colour variation. To measure the colour variation (peptide uptake), the wavelength at which the peptide absorbs was first determined. The peptide in solution was scanned over a wide wavelength range and the wavelength at which maximal absorbance occurs was identified at 555 nm. Colour variation was then measured directly from the hair-peptide solution by monitoring

absorbance at 555 nm before and after treatment. Since the colour of the solution is due solely to the presence of unbound peptide, the quantity of bound peptide that has penetrated the hair can be determined from the difference in absorbance (at 555 nm). The % colour variation was calculated by using the following equation:

$$\text{Colour Variation (\%)} = \frac{Abs_{initial} - Abs_{final}}{Abs_{initial}} \times 100$$

Zeta potential. Zeta potentials for the peptide solutions were measured by photon-correlation spectroscopy (PCS) using a Malvern zetasizer NS (Malvern Instruments) at $25.0 \pm 0.1^\circ\text{C}$. The instrument was routinely calibrated using a ± 66 mV latex standard.

Free thiol and disulphide bond determination. The amount of free thiol groups in the hair samples was determined spectrophotometrically using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) following a procedure adapted from Ellman (1959) for this purpose. Briefly, 10 mg of hair samples were added to 5 mL of 0.5 M PB, pH 8.0, containing 100 μL of Ellman's reagent solution (4 mg/mL) in the same buffer. The samples were mixed, and incubated for 1 h at room temperature in dark. Absorbance was then measured at a wavelength of 412 nm, and the amount of free thiol groups were determined from a calibration curve obtained using L-cysteine standards.

To determine the total amount of sulphur, a similar reaction with Ellman's reagent was performed after complete reduction of disulphide bonds in the hair samples using sodium borohydride (NaBH_4) (Hansen et al. 2007). Complete disulphide reduction was performed as follows: 10 mg of hair samples were hydrated in 350 μL of water for 10 min, then 150 μL of 0.05 M Tris buffer, pH 6.8 and 1.0 mL of freshly prepared 4% (w/v in 0.2 M NaOH) NaBH_4 solution were added. Samples were then incubated for 1 h in a shaking water-bath at $37 \pm 0.5^\circ\text{C}$. After the reaction, residual NaBH_4 was inactivated by addition of 200 μL of 5 M HCl under agitation for 10 min. The pH of the resulting reaction mixture was adjusted to 8.0 with 2 mL of 1 M PB, pH 8.0, and 100 μL of Ellman's reagent (4 mg/mL) was added to these mixtures and incubated for 15 min at room temperature. Absorbance at 412 nm was measured, and thiol groups were determined as described above.

The number of disulphide bonds present in the hair samples was calculated by subtracting the amount of free thiol groups present before NaBH_4 reduction from the amount of free thiols present after NaBH_4 treatment.

Fluorescence microscopy. Transversal cuts of the hair fibre samples were analysed by fluorescence microscopy.

Hair fibres were embedded into an epoxy resin, and 15 μm transversal cuts of the fibres were prepared using a microtome (Microtome Leitz). Fibre cross sections were analysed using a LEICA DM 5000B fluorescence microscope (Leica) at a magnification of $40\times$. All fluorescence microscopy images were recorded using identical filter, exposure, brightness and gain settings. The most representative images were chosen.

Mechanical properties. Mechanical properties of the hair samples were evaluated following guidelines outlined in ASTM D1445-95 for fibre tensile testing. Measurements were performed using an Instron 4505 tensile tester, with a load cell capacity of 2.5 N. For each measurement, 10 single hair fibres were randomly taken from the hair tress. Each hair was individually mounted in the tensile jig by means of a paper device with a fixed gauge length of 20 mm. Before the test, the paper device was slashed. Samples were kept under the same conditions before measurements. Measurements were performed at a constant rate of 1.5 mm/min until breakage. For each hair, the applied load against extension was recorded, assuming an average mean diameter of 70 μm (previously measured by light microscopy on transversal cut hair samples). Data were converted to stress (load/unit area) against strain (% extension).

Scanning electron microscopy. Hair fibres were taken randomly from the hair tress and mounted onto aluminium stubs using conductive carbon adhesive tape, and sputter coated from a gold/palladium leaf source to impart conductivity to the surface of the sample. The thickness of the coating was approximately 10 nm. Samples were studied using a NOVA Nano SEM 200 FEI. The SEM microscope was operated at 10 kV, and samples were viewed at a working distance of 8 mm at $10\,000\times$ magnification.

Differential scanning calorimetry. Thermal studies of the hair samples were conducted using a power-compensated Differential scanning calorimetry (DSC) instrument (DSC-7, Perkin Elmer), and pressure-resistant (25 bar), stainless steel, large-volume capsules, at a temperature range from 50 to 250°C (heating rate: $5^\circ\text{C}/\text{min}$, sample weight: 7–8 mg). The DSC device was calibrated using high purity indium and palmitic acid. Samples were stored at selected levels of humidity (RH of 45%) and temperature (20 – 22°C) for 24 h prior to DSC analysis. Each test group was measured in triplicate and mean values are reported.

Data analysis. Results were analysed using Graph Pad Prism 5.04 (Graph Pad Software, San Diego, CA). Statistical significances were determined using

a one way ANOVA, followed by the Dunnett post-hoc test or, alternatively, by the unpaired two-tailed Student's *t* test. *P* values ≤ 0.05 were considered to be statistically significant (expressed in the figures with asterisks [*]: **p* < 0.05, significant; ***p* < 0.01, very significant; ****p* < 0.001, extremely significant).

Results and discussion

Engineered peptides

The desire for healthy, beautiful hair has created a huge industry for the development of hair care products that improve its look and feel. However, commonly used chemical methods for bleaching hair can alter its properties, resulting in hair fibre damage (Gray 2001; Bolduc & Shapiro 2001; Dawber 1996). Therefore, new products able to counteract these problems are required. The peptides used in the present work contain cysteine residues and were developed to act as PDI substrates (Table I), for use in hair treatments designed to recover the properties of bleach-damaged hair following application of a traditional bleaching treatment.

The KP peptide was based on a fraction of the amino acid sequence of keratin type II cuticular Hb5, a protein that in humans is encoded by the KRT85 gene (Rogers et al. 1997; Koehn et al. 2010). A 13-amino acid fragment of this protein that comprises two cysteine residues separated by four other amino acids was then selected and chemically synthesized. Based on its origin, we hypothesized that this KP peptide would have affinity toward hair surfaces and the ability to restore damaged cuticles. In addition, because it is based on type II alpha-keratin, the KP peptide might possess inherent resilience and

pliability (Coulombe et al. 2004), which could be transferred to the hair fibres.

The SPB peptide was based on a fraction of the amino acid sequence of a pulmonary-associated surfactant protein B from mammalian lungs (Moore et al. 1992; Pilot-Matias et al. 1989). Diffusion of compounds into hair is usually hindered by their hydrophobic lipid layer. This lipid layer covers the entire hair surface, and is bound by thio-ester linkages to hair proteins (Breakspear et al. 2005). Surfactant proteins from mammalian lungs are able to recognize and interact with lipids, and peptide fragments based on these proteins have been used to increase the penetration and resistance of several compounds into hair (Cavaco-Paulo et al. 2007). Such properties make SPB peptides new and interesting candidates for use in hair care products.

The molecular properties of the synthesized peptides were visualized using the molecular visualisation program PyMol v1.4, which enables creation of structural models of peptides based on their amino acid sequences (Can et al. 2006). This program was used to visualize our peptide structures *in vacuo*, and to identify major differences in their surface charge and structure. Based on this analysis, both peptides were found to be amphipathic, a property which increases their affinity for biological membranes (Bessalle et al. 1993; Wimmer et al. 2005). Furthermore, KP appears to be mainly anionic, with a dominant negatively charged surface (red), while SPB is mainly cationic, with a dominant positively charged surface (blue) (Figure 1).

A similar trend was observed by comparison of the isoelectric points (pI) of the two peptides; a characteristic which may affect the affinity of a peptide towards hair. The pI is the pH at which the net charge

Figure 1. Peptides surface charge analysis created using the program PyMol (v1.4). Representation of the amino acid sequence and structure of (A) KP(X₃CX₅CX₃) and (B) SPB (XCX₇), from the N- to C-terminal. Red denotes negatively charged residues and blue denotes positively charged residues.

of the peptide is zero (i.e. the number of positive and negative amino acid residues is equal) (Franks & Meagher 2003). Thus, at pH 7.5 (the pH of the treatment) each peptide will possess a different net charge: the SPB peptide has a net positive charge because its pI is < 7.5 ; while the KP peptide has a net negative charge, because its pI is > 7.5 . This result was confirmed by zeta potential values (Table I).

Interestingly, the isoelectric point of human hair has been reported to be near 3.7 (Wilkerson 1935; Regismond et al. 1999), indicating that hair possesses a net negative surface charge under most hair treatment conditions. Thus, because electrostatic interactions are believed to play a significant role in hair adsorption mechanisms, we expected that the positively charged SPB peptide would easily adsorb onto the hair surface, while the negatively charged KP peptide would not.

Hair bleaching

Chemical bleaching is commonly used in cosmetics, and is applied to give hair a lighter look or, more often, to prepare it for dyeing. Bleach-based lightening treatments have the advantage of being able to directly modify the characteristics of melanin pigments, generally yielding hair with a lighter shade than the original colour (Zviak & Milléquant 2005). However, repetitive applications of bleach can modify the properties of hair along the cuticle and damage the hair fibre (Gray 2001; Bolduc & Shapiro 2001; Dawber 1996).

Figure 2 shows SEM images recorded for untreated (a) and over-bleached hair (b). As can be seen, untreated hair has complete cuticles with no

holes; while, in contrast, over-bleached hair has less visible cuticle layers (five layers are visible in virgin hair vs. three layers in over-bleached hair over the same area) with more and pronounced holes and debris fragments along the layers. Thus, chemical bleaching has destroyed the edges of the cuticle, releasing small cuticle fragments along the hair shaft.

PDI-catalysed peptide binding to over-bleached hair

Peptide uptake by the hair, which is a measure of peptide affinity, was determined using the colour variation method as described. As hypothesized above, the positively charged SPB displayed a higher affinity towards the hair samples than the negatively charged KP peptide (Figure 3). Therefore, initial peptide uptake seems to be dependent on the peptide isoelectric point, highlighting the importance of electrostatic interactions in hair treatments.

Nevertheless, the presence of PDI in the KP formulation solution promoted higher colour variation, indicating that PDI might catalyse covalent linkages between the KP peptide and hair. In contrast, the application of PDI together with the SPB peptide actually decreased peptide uptake (Figure 3). One possible explanation for this could be that PDI catalyses a conformational change in the SPB peptide, resulting in a decreased zone of contact between the peptide and the hair sample.

After the treatments, loosely bound peptides were washed off, and remaining tightly bound peptides (at the hair surface and/or cortex) were visualized by fluorescence microscopy. Although this method does not provide quantitative information on the amount

Figure 2. SEM images of normal virgin hair (A) and over-bleached hair (B). The bar represents 5 µm.

Figure 3. Uptake of peptides by over-bleached hair treated with or without PDI was measured by the colour variation method, in bath treatment solution at 555 nm.

of peptide bound, or details of the binding mechanism, fluorescence microscopy can confirm the presence of bound peptide on the hair samples.

Because both peptides were covalently labelled with a fluorescent dye, it was possible to qualitatively visualize the peptides that remained on the hair. A

similar approach was reported by Regismond and co-workers (1999). Figure 4 shows images of transverse cut hair fibres for unaltered human hair (VG), over-bleached hair treated with peptides ($8 \times$ B-KP; $8 \times$ B-SPB) and over-bleached hair treated with peptides in the presence of PDI ($8 \times$ B-KP-PDI; $8 \times$ B-SPB-PDI).

The intensity of the layer around the hair cuticle in VG hair treated with SPB is greater than in VG hair treated with KP (Figure 4 (B and C)), corroborating the higher affinity of SPB towards human hair (Figure 3, Table I). However, as can be seen in the fluorescence microscopy images, application of the peptides to chemically damaged hair resulted in increased penetration of both SPB and KP (Figure 4 (E and F)).

These results suggest that the presence of hair damage is a more important determinant of peptide attachment and affinity than the isoelectric point of the peptide. Over-bleached hair is believed to possess different physico-chemical properties than unaltered hair. For example, the surface charge of hair fibres depends on several factors, such as: microstructure, porosity, specific surface area, fibre swelling capacity

Figure 4. Fluorescence images of human hair cross sections from unaltered human hair (A, B and C) over bleached hair (D, E and F) and over bleached hair with PDI (G, H and I) along peptide treatment. KP and SPB were used to treat all fibres. Bars indicate 50 μ m.

and the interaction energy between the fibre and the solution (Barba et al. 2009). Because bleaching changes some of these factors (Zviak & Milléquant 2005), the surface charge of hair fibres following several rounds of bleaching is expected to be altered, thus explaining the differences in peptide interaction affinity observed in the present study. Moreover, the SEM images (Figure 2) demonstrate that bleaching results in hair cuticle damage, including the appearance of cracks, holes and cuticle lifting. This damage could explain the higher peptide penetration observed following over-bleaching, and increased peptide attachment to the hair (discussed in the next section). In fact, previous studies indicate that, for interactions which are primarily dependent on electrostatics, molecule adsorption on damaged hair increases with decreasing molecular weight (Mw) (Faucher & Goddard 1976).

Interestingly, the presence of PDI appears to improve the penetration of KP and SPB inside the fibre cortex, as previously reported for KP (Fernandes et al. 2011); while both peptides were bound at the cuticle surface in control experiments (KP and SPB without PDI) (Figure 4 (E and F) vs. (H and I)). In addition to penetrating the cortex, PDI probably promotes the formation of stronger linkages between the cysteine-containing peptides and the hair, which are resistant to washing. Using the approach of Nagy and co-workers (Nagy et al. 2007), quantitative determination of the amount of disulphide bonds in hair samples treated with peptides in the presence and absence of PDI was performed.

A higher free thiol content was found on hair treated with KP than hair treated with SPB, before complete disulfide reduction with NaBH_4 . KP possesses two cysteine residues, separated by five amino acid residues (Table I); thus it is possible that, in some cases, only one of these cysteine residues is involved in disulphide bond formation with cysteines from the hair (in presence of PDI), while the other remains free and is detectable by Ellman's reagent (Table II). In addition, in the absence of PDI, more

free thiol groups were observed in hair treated with KP than in hair treated with SPB (Table II). Due to its positive charges, the N-terminal of SPB possesses high affinity to hair (Figure 1) and when applied without PDI, electrostatic interactions are believed to play a major role in hair/peptide interaction. The cysteine residue is the second amino acid present in the sequence of this peptide (Figure 1), so if peptide binds to hair through the N-terminal, no free thiol group is detectable by Ellman's reagents, explaining the fact that free thiol content of $8 \times \text{B-SPB}$ is not increased when compared to that of $8 \times \text{B}$.

Regarding disulphide bonds, higher quantities were detected in hair treated with peptides in the presence of PDI (Table II). In the case of KP, PDI promoted an increase in disulphide bonds from 1.6 to 2.7 $\mu\text{M}/\text{mg}$ of hair. Similarly, SPB treatment in the presence of PDI resulted in an increase from 1.46 to 3.27 $\mu\text{M}/\text{mg}$ of hair. These results support the hypothesis that the PDI enzyme is able to catalyse formation of covalent linkages via creation of disulphide bonds between the cysteine-containing peptides and cysteines on the hair samples.

Recovery of the mechanical and thermal properties of over-bleached hair

The mechanisms by which hair care products interact with and alter the chemical and physical properties of hair are of significant interest to the cosmetics industry, since these properties are closely tied to product performance.

Because KP and SPB have affinity toward over-bleached hair and can be covalently bound in the presence of PDI, we wanted to investigate whether their application influenced the mechanical strength and thermal properties of bleach damaged hair. Therefore, the physical and thermal properties of damaged hair fibres before and after peptide treatment were evaluated. The Young's modulus (stiffness) and ultimate tensile strength of the hair samples are presented in Figure 5. Because determination of these parameters is prone to variation, depending on the method of measurement and the part and type of hair measured, a set of 10 different hair samples was examined in order to validate the results. The damage imparted by consecutive chemical bleaching treatments resulted in a loss of resistance. In addition, a statistically significant loss of stiffness ($**p < 0.01$) was observed following consecutive chemical bleaching treatments. However, although a decrease in ultimate tensile strength was also observed, this result was not statistically significant. Nevertheless, these results agree with previous studies, which reported that bleaching treatments decreased the mechanical strength of hair by approximately 25% (Erik et al. 2008).

Table II. Quantification of free thiol groups(-SH) and disulphide bonds of over-bleached hair treated with peptides in the presence and absence of protein disulphide isomerase (PDI).

	Free thiol -SH ($\mu\text{M}/\text{mg}$ of hair)	S-S bonds ($\mu\text{M}/\text{mg}$ of hair)
$8 \times \text{Bleaching}$	1.6 ± 0.3	1.3 ± 0.1
$8 \times \text{Bleaching and KP}$	3.4 ± 0.6	1.6 ± 0.1
$8 \times \text{Bleaching and KP + PDI}$	2.6 ± 0.6	2.7 ± 0.1
$8 \times \text{Bleaching and SPB}$	1.65 ± 0.08	1.46 ± 0.08
$8 \times \text{Bleaching and SPB + PDI}$	0.04 ± 0.01	3.27 ± 0.01

Figure 5. Mechanical resistance parameters: (A) Young's modulus and (B) tensile strength of virgin (VG) and over-bleached ($8 \times B$) hair, and over-bleached hair treated with peptides (KP and SPB) in the presence of PDI. Data represents the mean $\pm$ SD of 10 independent experiments.

Application of both peptides in the presence of PDI resulted in recovery of the hair's mechanical properties. Specifically, application of KP was associated with a statistically significant recovery of both Young's modulus ($***p < 0.001$) and ultimate tensile strength ($*p < 0.05$); while application of SPB significantly improved hair stiffness ($*p < 0.05$). Repeated bleaching treatments induced hair cuticular damage, and consequently, altered the mechanical properties of the hair. Thus, PDI induced

penetration of the peptides into the hair cuticle may explain the improved mechanical properties observed. Although SPB has a higher overall affinity for hair, KP is a fragment of a cuticular keratin protein, and may be better able to recover cuticle structure by filling in holes in the cuticles of damaged hair penetrating inside the hair fibres.

It is widely accepted that both the mechanical and thermal properties of hair are strongly influenced by their immediate chemical environment, including hair treatment formulations (Wortmann et al. 2002, 2006, 2008). Therefore, to corroborate our findings that PDI-catalysed peptide treatments recover the mechanical properties of over-bleached hair, we performed DSC measurements, to assess the denaturation of human hair keratin following peptide treatment. Although human hair exhibits complex morphology as an α -keratin fibre, for the purposes of thermal analysis it can be considered to be a filament/matrix-composite, as originally proposed by Feughelman (1959, 1997). In this model, the α -helical fraction of the intermediate filaments (IF) comprise the crystalline filament phase, which is embedded in an amorphous matrix, characterized by the presence of intermediate filament associated proteins (IFAPs), among other amorphous morphological components.

Table III shows the temperatures and enthalpies associated with the removal of loosely bound water, as well as the temperature and enthalpy values for α -helix keratin denaturation. Thermal transitions in keratin fibres are strongly affected by the amount of bound water and a broad endothermic signal, related to the removal of loosely bound water, appears at around 100°C (Humphries et al. 1972). Interestingly, a decrease in the temperature and enthalpy at which this removal of water occurs was observed in chemically damaged hair; possibly due to the presence of hair surface damage caused by over-bleaching. Furthermore, the holes and partial removal of cuticles observed by SEM (Figure 2) on bleach damaged hair may facilitate a faster pathway for water removal, thus lowering the temperature and enthalpy values versus virgin hair, whose surface

Table III. Temperatures of water removal (T_{dw}) and α -helix denaturation ($T_{d\alpha}$), and enthalpies of water removal (ΔH_w) and α -helix denaturation (ΔH_α) of over-bleached hair ($8 \times$ Bleaching), virgin hair (VG) and over-bleached treated hair, with and without PDI.

	T_{dw} ($^\circ\text{C}$) ^a	ΔH_w (J/g) ^a	$T_{d\alpha}$ ($^\circ\text{C}$) ^a	ΔH_α (J/g) ^a
Virgin hair (VG)	92.8 ± 0.8	244.1 ± 4.4	229.3 ± 0.7	6.8 ± 0.2
$8 \times$ Bleaching	86.5 ± 1.9	178.4 ± 18.5	230.6 ± 4.5	4.2 ± 1.1
$8 \times$ Bleaching and KP	93.7 ± 2.4	262.9 ± 11.8	229.0 ± 1.7	5.1 ± 0.5
$8 \times$ Bleaching and KP + PDI	116.2 ± 1.7	95.5 ± 9.8	241.0 ± 2.6	9.9 ± 1.5
$8 \times$ Bleaching and SPB	89.4 ± 0.3	226.6 ± 9.5	235.7 ± 5.3	6.6 ± 2.4
$8 \times$ Bleaching and SPB + PDI	128.0 ± 4.1	54.4 ± 8.2	241.0 ± 7.8	10.3 ± 3.1

^aData presents mean $\pm$ SD (standard deviation) from a minimum of three experiments.

was not damaged. Moreover, incorporation of both peptides on this chemically-damaged hair, in the absence of PDI, increased the temperature and enthalpy of water removal (Table III), probably due to the ability of these peptides to cover the hair surface. Because of this, less water adsorption on the hair surface is thought to occur, and therefore, water removal in the presence of surface bound peptide is delayed to higher temperatures. PDI was found to significantly increase the temperature at which hair surface water is removed, again corroborating the evidence that PDI induces the attachment of peptides onto damaged hair. The enthalpy, however, was significantly decreased, indicating that a lower energy was needed to remove the surface bound water. Although these results appear to be contradictory, a possible explanation may be that, since PDI promotes the incorporation of peptides inside the hair cortex, less peptide would cover the hair surface, shifting the enthalpy to lower values.

The other important parameter analysed by DSC is the denaturation of the α -helical keratin fraction, which is characterized by a signal with a maximum temperature between 210 and 250°C (Monteiro et al. 2005). The α -helix denaturation temperatures ($T_d\alpha$) of chemically bleached hair fibres were similar to those obtained for virgin, non-damaged hair (Table III). However, after peptide treatment in the presence or absence of PDI, these α -helix denaturation temperatures shifted toward higher values (with the exception of hair treated with KP in the absence of PDI). Because the α -helical content of hair is thought to be strongly affected by chemical treatments (Wortmann 1993), investigations into the changes that results from different hair treatments would be informative.

In this study, the negative effects of repeated bleaching treatments and the positive effects of subsequent PDI-catalysed peptide treatments have been demonstrated. Based on our results, it can be summarized that more energy is clearly needed to denature the α -keratin structure of healthy, virgin hair or hair treated with peptides, than over-bleached, damaged hair.

Conclusions

The conventional chemical treatments have detrimental effects on the mechanical and thermal properties of hair, which require the use of mild, enzyme-based hair formulations for recovery of these lost properties. In the present study, surfactant- and keratin-based peptides were applied to chemically over-bleached hair, and their ability to improve its mechanical and thermal properties was

investigated. Since both peptides contain cysteine residues, the application of PDI promoted the covalent attachment of the peptides onto hair via disulphide bonds increasing their penetration into the hair cortex. Finally, the peptide treatments tested in the present study induced a significant recovery in stiffness and tensile strength in bleach damaged hair along with an increase in the α -helix denaturation enthalpy of the treated hair.

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