Strategies for improved hair binding: Keratin fractions and the impact of cationic substructures

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ABSTRACT

Keratin extracts and hydrolysates from varying sources, their chemical modifications and compositions thereof have shown potential in the restoration of hair properties. Within this study on reactivity of thiol groups and the shielding effect of anionic charges the binding of keratin-associated proteins (KAP) and α-keratins (Ker) extracted from human hair to natural and permed hair fibers was evaluated. Selectively extracted KAP and Ker were preactivated with 6-mercaptopentanamide in a quantity of 194 ± 21 μmol/g for KAP and 169 ± 27 μmol/g for Ker resulting in 1.9- and 1.4-fold enhanced binding to natural hair, respectively. The amount of accumulated Ker on hair fibers was furthermore increased by 1.7-fold in presence of 25 mM L-arginine. Perming of hair impaired binding characteristics of Ker with negligible effects for preactivation, whereas unmodified and preactivated KAP showed results comparable to natural hair. Strongly enhanced penetrability after perming was reflected by the mean penetration depth for fluorescein of 25 μm compared to 5 μm for natural fibers.

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1. Introduction

Besides environmental influences like UV-light, cosmetic treatments of hair such as bleaching, dyeing, permanent waving, or grooming count to the main causes of hair damage associated with alterations in the surface architecture of the highly keratinized fibers [1]. The extent of hair damage can be characterized focusing on physical properties such as mechanical strength and moisture content or morphological changes. Moreover, several attempts were made to identify variations in different structural components of hair fibers [1–3].

Displaying the main proteinaceous constituent with about 80% of total hair mass, keratin gives the fiber its distinct mechanical strength, flexibility and durability due to its high cysteine content and thus pronounced ability to form inter- and intramolecular disulfide bridges [4,5]. Hard keratins in human hair can be distinguished in highly cross-linked α-keratins forming intermediate filaments that are further organized in micro- and macro-fibrils, β-keraatin located in the cuticle as well as γ-keratins. The latter are also known as matrix proteins or keratin-associated proteins (KAP) and form the non-filamentous matrix [6].

To compensate the consequences of hair damage, keratin proteins and peptides from different origin were investigated for their potential to restore the hair structure and maintain surface as well as mechanical properties [7–10]. In the course of studies for keratin binding to hair the molecular mass of S-sulfonated keratin was shown to affect film forming and therefore moisturizing properties of the protein as well as the improvement of mechanical strength of bleached and permend fibers [7]. Besides these size dependent effects, hydrophobic interactions and moreover electrostatic repulsion from anionic hair fiber are known to have an impact on accumulation of substances [11].

In order to further intensify the binding of proteins, the effect of last-mentioned non-covalent interactions was supplemented by different approaches to form covalent bonds ranging from enzyme mediated binding to the introduction of an activated substructure to keratin [7,9]. In this context, a recent study demonstrated the successful protection of thiol groups from oxidation and enhanced reactivity towards covalent attachment to superficial hair structures by means of a mercaptopropyridine derivative. The introduction of the negatively charged nicotinic acid structure as a leaving group resulted in a significant increase of bound keratin to natural and bleached hair [12].

Encouraged by these promising findings, it was the objective of this study to assess parameters possibly further enhancing keratin accumulation on the fiber surface providing deeper insights in its binding characteristics. Pursuing an investigation of the effect of charge originating from the S-protecting ligand, preactivation of proteins with 6-mercaptonicotinamide was performed. Moreover, combination with the charge shielding additive L-arginine was intended to minimize electrostatic repulsion from the fiber surface. To further elucidate hair binding properties of keratin extracted from human hair, fractions containing predominantly KAP or α-keratins were separated. In order to broaden the applicability of results, the effect of surface charge as
well as preactivation of thiol groups and molecular mass of keratin on binding to hair was examined for natural and permed fibers possessing fundamentally altered surface characteristics.

2. Materials and methods

2.1. Materials

Sodium dodecyl sulfate (SDS), urea, thiourea, 2-mercaptoethanol (2-ME), hydrogen peroxide solution (30 wt% in water), Rotiphorese® Gel A (30% acrylamide stock solution), Rotiphorese® Gel B (2% bisacrylamide stock solution) and L-arginine x HCl were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). PageRuler™ Unstained Protein Ladder and glucosamine x HCl were obtained from Thermo Fisher Scientific (Massachusetts, USA). \(N,N,N',N'-\text{tetramethyl-}
\text{ethylene diamine (TEMED), ammonium persulfate (APS) and}
\text{Coomassie Brilliant Blue G-250 were supplied by Serve Electrophoresis}
\text{GmbH (Heidelberg, Germany). 1,4-Dithiothreitol (DTT) was purchased}
\text{from Roche Diagnostics GmbH (Mannheim, Germany). Fluorescein iso-}
\text{thiocyanate Isomer 1 (FITC), 6-chloronicotinamide, sodium borohy-}
\text{dride, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), L-glutathione}
\text{reduced (GSH), thioglycolic acid (TGA), fluorescein sodium and all}
\text{other salts and solvents were purchased from Sigma-Aldrich (Vienna,}
\text{Austria).}

2.2. Selective extraction of keratin-associated proteins (KAP) and \(\alpha\)-keratins

To facilitate a differentiated characterization of keratin-associated proteins (KAP) and \(\alpha\)-keratins (Ker) a separated extraction of these protein fractions was used according to a previously described method with slight adaptations [13]. Therefore, 6.25 g of Caucasian hair that did not perceive any treatments such as hair dyes, bleaching or perms was washed with 0.5% SDS solution, rinsed with demineralized water and dried at room temperature. For the extraction of KAP the hair was cut to a length of 1 cm and mixed at a concentration of 50 mg/mL with the extraction medium containing 8 M urea, 5% (v/v) 2-ME and 35% (v/v) ethanol in 25 mM Tris buffer pH 9.5. After incubating for 72 h at 50 °C under reflux the KAP extract was separated from remaining hair fragments by filtration through a fine metal sieve and subsequent centrifugation at 15,000 \(\times g\) for 10 min at 23 °C (Sigma 3-18KS, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The remaining KAP extracted hair was washed twice with 125 mL of demineralized water. After the removal of excess water using a fine metal sieve the hair fragments were suspended in Ker extraction medium containing 2.6 M thiourea, 5 M urea and 5% (v/v) 2-ME in Tris buffer 25 mM pH 8.5 at a concentration of 60 mg/mL. The mixture was incubated at 50 °C for 24 h and subsequently centrifuged under the same conditions as applied for the KAP extract. The obtained Ker and KAP extracts were purified by exhaustive dialysis against demineralized water at 19 °C in the dark using dialysis tubes with a MWCO of 3.5 kDa (regenerated cellulose, Carl Roth GmbH & Co. KG) and lyophilized for 42 h (Christ Gamma 1–16 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

Keratin extracted according to the Shindai method served as control [14]. Therefore, hair was washed and cut in the same way as described above. The dried hair was dispersed in the extraction medium at a concentration of 40 mg/mL containing 2.6 M thiourea, 5 M urea, 5% (v/v) 2-ME in 25 mM Tris buffer pH 8.5 and heated to 50 °C for 72 h under stirring. Afterwards the obtained Shindai extract was centrifuged, purified and dried similarly to KAP and Ker extracts.

2.3. Reduction and preactivation with 6-mercaptonicotinamide (6-MNA)

The chemical modification of extracted KAP and Ker was performed according to a previously described method with slight adaptations due to the solubility characteristics of the employed protecting group (Fig. 1) [12]. Therefore, 200 mg of the respective unmodified protein was suspended in 50 mL of Tris buffer 25 mM pH 8.5 with the help of ultrasonication. Then, NaBH4 in a final concentration of 1% (w/v) was added to the suspension and stirred for 1 h at room temperature. To inactivate the excess of reducing agent 5 mL of 5 M HCl was added dropwise and the precipitated protein was separated by filtration (cellulose, particle retention 5–13 \(\mu\)m). The obtained precipitate was washed with 10 mM HCl and suspended in 6 mL of demineralized water.

![Fig. 1. Reaction scheme of synthesis of KAP-MNA and Ker-MNA. Disulfide bonds in KAP/Ker were reduced by NaBH4 (A). Free thiol groups of reduced KAP (KAP-SH) and Ker (Ker-SH) were preactivated with 6-MNA (B).](image-url)
Subsequently, 6-mercaptopicolinic acid (6-MNA) synthesized as previously described and oxidized to its dimeric form 6,6′-dithionicotinamide (6,6′-DTNA) with hydrogen peroxide [15] served to preactivate the resulting free thiol groups. Therefore, 60 mg of 6,6′-DTNA were dissolved in 30 mL of DMSO and the aqueous protein dispersion was added dropwise at pH 8 to 9. The reaction mixture was stirred at room temperature for 6 h. Then, the 3-fold volume of acetone was added to the mixture. To remove unreacted 6,6′-DTNA and 6-MNA the precipitated protein was separated by filtration (cel lulose, particle retention 5–13 μm), redispersed in 60 mL of DMSO and again precipitated in the 3-fold volume of acetone. After repeated filtration the precipitate was dispersed in deionized water and further purified by exhaustive dialysis using dialysis tubes with a MWCO of 3.5 kDa (regenerated cellulose, Carl Roth GmbH & Co. KG). The obtained product was lyophilized (Christ Gamma 1-16 LSC) and stored dry until further use.

2.4. Chemical characterization

Unmodified as well as preactivated KAP and Ker derivatives were analyzed photometrically for their content of free thiol groups by means of DTNB in a final concentration of 0.015% in 0.5 M phosphate buffer pH 8.0 at a wavelength of 450 nm [16]. Similarly, the total amount of thiol moieties was determined after reduction with NaBH4 [17]. The amount of immobilized 6-MNA was quantified photometrically at a wavelength of 312 nm after cleavage of disulfide bonds with GSH in a final concentration of 0.1% (w/v) in 0.25 M phosphate buffer pH 8.0 [15].

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli [18] using a gel with a crosslinking ratio of 2.7% and a thickness of 2.5.

2.6. Zeta potential measurements

Zeta potential of extracted KAP and Ker in presence of increasing amounts of additives with cationic structures was measured by means of NICOMP™ 380 ZLS PSS (Santa Barbara, California, USA) applying an electric field strength of 5 V/cm for 3 min at 23 °C. Dispersions of KAP and Ker in a concentration of 0.2 mg/mL were adjusted to pH 8 and homogenized by ultrasonication. L-arginine as well as glucosamine were added in increasing concentrations and the zeta potential of the dispersion was measured. Moreover, the isoelectric point (IEP) of unmodified as well as preactivated KAP and Ker was determined according to a previously described method [12].

2.7. Hair treatment

Untreated hair used for binding studies was washed with 0.5% SDS, rinsed with deionized water and air-dried. For perming treatment, hair was immersed in 8% thioglycolate adjusted to pH 9.0 with ammonia at a concentration of 25 mg/mL for 3 h under continuous shaking. After washing with deionized water for 5 min the hair was placed in a neutralizing solution containing 2.5% H2O2 at pH 3.0 for 30 min. The permed hair was rinsed with deionized water and dried on air [7].

2.8. Labeling with FITC

For labeling of unmodified as well as preactivated KAP and Ker derivatives following a previously described method with slight adaptations [12] the respective proteins were dispersed in 100 mM carbonate buffer pH 9.0 in a concentration of 20 mg/mL. FITC reacting selectively with ε- and N-terminal amines was added dropwise in a concentration of 6.67 mg/mL in ethanol in a mass ratio of protein/FITC of 40/1. The suspension was stirred in the dark for 4 h at 4 °C and dialyzed (MWCO of 3.5 kDa, regenerated cellulose, Carl Roth GmbH & Co. KG) against 10 mM phosphate buffer pH 7.4 until the absorption of the dialysis water at 495 nm was below 0.003 [19]. The purified proteins were lyophilized (Christ Gamma 1-16 LSC) and stored at 4 °C in the dark in airtight containers.

2.9. Solubility studies

Solubility of KAP and Ker derivatives was determined based on the fluorescence intensity of the respective dispersions before and after removal of undissolved protein. Therefore, KAP and Ker samples were suspended in 100 mM phosphate buffer pH 8.0 in a concentration of 0.2 mg/mL containing increasing amounts of L-arginine and finely dispersed using an ultrasonication stick (UP200H, Hielscher Ultrasonics GmbH, Teltow, Germany). The obtained suspensions were centrifuged at 15,000 × g (Sigma 3-18KS) and the supernatant was removed.

Samples from the suspension before centrifugation as well as from the clear supernatant were diluted, 1:1 with 5 M NaOH and heated to 60 °C for 15 min under shaking and protected from light to completely solubilize undissolved parts of protein. For reasons of comparability also samples of solubilized fractions were treated in the same way. The fluorescence intensity of samples was measured at an excitation wavelength of 495 nm and emission wavelength of 534 nm (TECAN Spark®, Tecan Group Ltd., Männedorf, Switzerland). The amount of solubilized protein was calculated as percentage of fluorescence remaining after centrifugation under consideration of the fluorescence of blank solutions containing the respective unlabeled protein.

2.10. Hair binding studies

Protein samples for hair binding studies were prepared according to 2.9 in a final concentration of 0.04 mg/mL in phosphate buffer 100 mM pH 8.0 with varying concentrations of L-arginine. 10 mg of untreated and permed hair was incubated for 30 min with 2.0 mL of the protein dispersion at room temperature in the dark under continuous shaking at 500 rpm. Then, samples of the supernatant were withdrawn, diluted 1:1 with NaOH 5 M and heated to 60 °C for 15 min. Protein dispersions treated in the same way but omitting the contact to hair fibers were taken as controls. All samples were analyzed at an excitation wavelength of 495 nm and emission wavelength of 534 nm (TECAN Spark®, Tecan Group Ltd., Männedorf, Switzerland). The amount of solubilized protein was calculated as percentage of fluorescence remaining after centrifugation under consideration of the fluorescence of blank solutions containing the respective unlabeled protein.

\[
\text{protein bound to hair (mg%) = } \frac{\text{fluorescence (control) - fluorescence (hair)}}{\text{fluorescence (control)}} \times 100
\]

where fluorescence (hair) is the fluorescence of samples after incubation with hair and fluorescence (control) the fluorescence of the control. For the calculation blanks were subtracted comprising the equivalent buffer after incubation with hair and without contact to hair fibers.
2.11. Confocal microscopy

Samples of untreated and permed hair having been incubated with unmodified and preactivated KAP and Ker derivatives as described in 2.10 were washed with demineralized water and dried in the dark at room temperature. As a control for the penetration into hair fibers, untreated and permed hair was incubated with sodium fluorescein in a concentration of 4.0 μg/mL in phosphate buffer 100 mM pH 8.0 for 30 min under continuous shaking in the dark. To remove excess sodium fluorescein the hair was washed with demineralized water and dried at room temperature protected from light. Single hair fibers were mounted on microscope slides and immersed in glycerol. Samples of virgin and permed hair were imaged by means of a Leica SP8 confocal laser scanning microscope (Leica, Wetzlar, Germany) with a 63 × 1.3 NA glycerol objective applying uniform confocal settings. Imaging of the fluorescence contrast was done with a maximum number of 41 optical sections (z-stacks) that were spaced 1 μm apart in the z-axis. Using the 15 uppermost z-stacks, single in-focus projection images of the fiber surface and cross section images of all samples were generated by means of an ImageJ script as result of the projection of 10 longitudinal slices along the y-axis. Due to the employed ImageJ-script during post-processing of the image data, intensity values/pixel were displayed in red color. Being arbitrary definable, however, this choice of color does not affect the validity of results.

Table 1

|               | -SH [μmol/g] | -SH after reduction with NaBH\(_4\) [μmol/g] | 6-MNA [μmol/g] |
|---------------|--------------|---------------------------------------------|----------------|
| KAP           | 21.4 ± 7.8   | 2367.8 ± 311.5                              |                |
| Ker           | 36.7 ± 18.6  | 981.5 ± 148.7                               |                |
| KAP-MNA       | 10.0 ± 0.6   | 1578.0 ± 167.7                              | 193.6 ± 20.8   |
| Ker-MNA       | 9.9 ± 2.3    | 1097.9 ± 84.0                               | 169.0 ± 27.4   |

Fig. 3. Zeta potential of mixtures of Ker and glucosamine (●), Ker and L-arginine (○) as well as KAP and L-arginine (▲) at pH 8.0 (23 °C). Proteins were used in a concentration of 0.2 mg/mL. Depicted values are means of at least three experiments ± standard deviation.

Fig. 4. Solubility of KAP (●), Ker (○), KAP-MNA (▲) and Ker-MNA (△) in 100 mM phosphate buffer pH 8.0 with increasing concentration of L-arginine at a protein concentration of 0.2 mg/mL. Values represent data of at least three experiments ± standard deviation.

Fig. 5. Amount of KAP (white bars) and Ker (gray bars) bound to 10 mg of untreated hair starting from a concentration of 0.04 mg/mL in 100 mM phosphate buffer pH 8.0 with increasing concentrations of L-arginine. Displayed values are means of at least three experiments ± standard deviation. (**p ≤ 0.001).
In order to determine the fluorescein-molecule penetration depth into untreated and permmed hair fiber, respectively, a fully automatized approach as visualized in Fig. S1 was developed utilizing ImageJ and Python. First the orientation of the hair fiber was determined (1). Subsequently 20 cross sections, spaced 1 μm apart and perpendicular to the hair fiber orientation, were generated (2). As a measure of the fluorescein-molecule penetration depth the z-axis intensity-value distribution at ~3000 points along the x-axis of each cross-section was analyzed. The distance between the maximum intensity value at the hair fiber surface and a beforehand determined threshold value inside the hair along z-axis gave the penetration depth per point. The threshold value was defined as the intensity value of the average background auto-fluorescence value of the hair sample (3). For each sample three different hair samples were imaged at three different locations along the hair fiber, respectively, which gives a total of 5.4 \cdot 10^6 points/sample being used to determine the penetration depth distribution.

2.12. Statistical data analysis

Statistical data analysis was performed using student’s t-test to compare means of two groups and one-way ANOVA combined with Bonferroni post-hoc analysis to examine significance of differences between means of more than two groups. \( P < .05 \) was set as significant (*), \( P < .01 \) as very significant (**) and \( P < .001 \) as highly significant (***)

3. Results

3.1. Preparation of keratin derivatives

The successful extraction of KAP in presence of hydroxyl groups of ethanol and 2-ME inhibiting the dissociation of Ker from the hierarchical hair protein architecture [13] was shown via SDS-PAGE as displayed in Fig. 2. Moreover, a further increase in concentration of hydroxyl groups in the extraction medium in comparison to the underlying procedure [13] facilitated an isolation of matrix proteins with a molecular mass of 10–20 kDa in the KAP extract. Proteins with a molecular mass bigger than 25 kDa were extracted with α-keratins consisting of acidic (type Ia) and basic (type IIa) keratins with a molecular mass of 40–50 kDa and 55–65 kDa, respectively, displayed as Ker extract [13]. Beyond that, high molecular mass minor components with a molecular mass of 110–115 and 125–135 kDa that are extracted using Shindai method were also part of this Ker fraction [14].

Results for the chemical characterization of the obtained proteins and modified derivatives are shown in Table 1. Extracted KAP revealed a 2.4-fold higher thiol content in comparison to Ker which is in good agreement with literature [11,13]. After removal of the extraction medium both products showed precipitation due to the reformation of disulfide bonds. Lyophilized KAP appeared as compact powder, whereas Ker formed a cohesive voluminous lyophilizate. The amount of free thiol groups after reduction was 282.0 ± 11.2 μmol/g and 499.2 ± 113.0 μmol/g protein for KAP and Ker, respectively. However, the extent of S-protection in the mild oxidative environment of ~80% DMSO [20] was restricted for both products in comparison to preactivation with 2-mercaptonicotinic acid in water [12].

Cleavage of disulfide bonds and subsequent introduction of 6-MNA as non-ionic protecting group caused a decrease in the total amount of thiol moieties for KAP. A control treated in the same way as preactivated proteins without addition of 6,6-DTN A (light gray bars) as well as KAP-MNA and Ker-MNA (dark gray bars) bound to untreated hair in 100 mM phosphate buffer pH 8.0 starting from a concentration of 0.04 mg/mL. Values are means of at least three experiments ± standard deviation. (*) \( P < 0.05 \), (***) \( P < 0.001 \).

3.2. Zeta potential measurements

The capability of employed additives to shield the negative surface charge of extracted proteins resulted in an approximation to neutral charge with increasing concentrations of glucosamine and L-arginine, respectively, as displayed in Fig. 3. In presence of the guanidinium group of L-arginine a plateau was reached at a concentration of 25 mM for KAP and Ker, whereas the addition of the amino monosaccharide glucosamine revealed a zeta potential close to zero starting from 100 mM. At higher concentrations of L-arginine a decrease of zeta potential was observed for KAP and Ker being not significantly different from its maxima at 25 and 50 mM followed by an approximation to 0 mV at 1000 mM. No significant difference in zeta potential of KAP and Ker in combination with L-arginine was observed throughout the investigated concentration range. According to these findings L-arginine was used as an additive in hair binding studies.

The higher content of aspartic and glutamic acid of Ker in comparison to the KAP fraction with little differences in the content of basic amino acids [13] may contribute to discrepancies in the IEP of protein fractions. Accordingly, KAP exhibited an IEP of 4.62 ± 0.06 and Ker of 4.36 ± 0.06 while preactivation with 6-MNA caused no significant changes in IEP for both fractions.

3.3. Solubility studies

Taking the composition of the respective protein fraction and chemical modification into account, the inherent limited solubility of keratins could be further differentiated as shown in Fig. 4. Revealing a significantly higher concentration of total thiols, KAP were observed to have an even lower solubility than Ker due to more pronounced crosslinking. Reduction of both protein fractions and subsequent introduction of 6-MNA possessing itself a limited solubility in water further decreased the proportion of solubilized KAP-MNA and Ker-MNA. The addition of L-arginine in increasing concentrations aimed to neutralize the surface charge of KAP and Ker did not cause substantial changes in solubility of unmodified keratin derivatives. A change in the trend of solubility was ob served for preactivated derivatives at a concentration of 250 mM with a drop for Ker-MNA at this concentration followed by an augmented solubility for KAP-MNA and Ker-MNA at 1000 mM. This observation is in agreement with alterations in zeta potential at the respective concentrations as described in 3.2. Variations in solubility of preactivated protein may be a consequence of the preferential interaction of L-arginine with
aromatic amino acids. Moreover, the formation of dimers and n-mers in aqueous solutions depending on the concentration of L-arginine and consequent loss in surface area may have an impact on interactions with the protein. Within a molecular dynamics study a strong decrease of surface area normalized to the number of L-arginine molecules was observed in a concentration range of 0–500 mM, whereas changes at higher concentration were less pronounced [21].

Aiming to compare protein conjugates with the observed fundamental differences in solubility, a working concentration of 0.04 mg/mL was chosen for hair binding displaying 20% of the starting concentration in solubility studies.

3.4. Hair binding studies

3.4.1. Binding to untreated hair

Though binding in a similar quantity, unmodified proteins of KAP and Ker fraction revealed a differentiated response to the addition of L-arginine during incubation with natural hair fibers. As displayed in Fig. 5, Ker fraction showed a maximum amount of attached protein at a concentration of L-arginine of 25 mM. This finding is in good accordance with the approximation of zeta potential to 0 mV and the observation of a plateau starting at this concentration (Fig. 3). By contrast, no significant improvement was observed for KAP showing that the zeta potential is not the only parameter that has to be taken into account. Molecular mass, film forming properties and thiol content of the protein fractions will likely also contribute to the overall binding properties. Higher amounts of L-arginine, however, may hinder proteins to bind due to a competitive effect with pronounced shielding of protein and hair surface.

The increase of hair binding caused by preactivation with 6-MNA was 1.9 fold for KAP-MNA and 1.4 fold for Ker-MNA as depicted in Fig. 6. This is in good agreement with a previous study assessing the binding of keratin extracted by Shindai method and preactivated with 2-mercaptanonic acid [12]. Reduction and subsequent washing
steps with DMSO of KAP designated as KAPcontrol lead to augmented binding to hair fibers, though being not significantly different from the untreated KAP. Likewise, no significant difference was observed for Kercontrol underlining the enhanced adhesion due to immobilization of G-MNA and not solely by synthesis conditions. The combination of both approaches of shielding negative surface charges by adding L-arginine and enhancing reactivity of thiols by preactivation did not show a synergistic effect (data not shown).

The resulting distribution of protein on the cuticle of untreated hair fibers was more evenly spread for Ker than for KAP with a pronounced accumulation along the cuticle edges for both fractions as shown in Fig. 7. The tendency to accumulate in bigger clusters after preactivation was observed for both protein fractions, whereas the addition of L-arginine had no visible effect on the distribution.

3.4.2. Binding to permed hair

Perming of hair strongly affected binding characteristics of unmodified and preactivated proteins as displayed in Fig. 8. While KAP binding was still significantly improved by preactivation, no significant difference was observed for preactivated Ker. Moreover, the amount of accumulated Ker was significantly lower than for untreated hair, whereas the effect of L-arginine was comparable to the results for untreated hair for both protein fractions. The decrease of KAP-MNA binding in presence of L-arginine is comparable to that of KAP binding to natural hair. The lacking response to the additive and possible shielding of the protein surface at higher concentrations might be even more pronounced due to the strongly enhanced reactivity of KAP-MNA.

The distribution of proteins was more consistent along the hair fiber in comparison to untreated hair with a less pronounced concentration at cuticular edges as shown in Figs. 9 and 10. Similar to the observed pattern on untreated hair this phenomenon was most distinct for Ker-MNA, visualizing changes in the cuticle of permed hair. The smooth and wavy edges of cuticular cells of untreated fibers seemed to be crumbled resulting in a more rectilinear sequence of dentated free edges. Owing to the harsh reductive alkaline conditions during perming, hair fibers possessed moreover deep axial folds.

Treatment of natural as well as permed hair samples with sodium fluorescein served to visualize differences in the penetrability of fibers. To facilitate a sufficient visualization, the employed concentration of fluorescent dye displayed the 50-fold concentration as compared to protein test solutions for keratin binding. While fluorescence images of untreated hair showed a differentiated staining of cuticle scales, permed fibers revealed an accumulation of the dye without recognizable pattern (Fig. 11). Cross sectional images further illustrate the deposition of dye on the intact cuticle layer of natural hair with scattered bright fluorescence regions in the cortex region associated to nuclear remnants [22]. By contrast, the synergistic reduction of disulfide bonds and the subsequent diffusion of TGA into the fiber under the employed alkaline conditions [23] facilitated a deeper gradual penetration of the dye with a lack of structural localization. Analysis of the distribution of fluorescein along the z-axis of the fiber revealed a mean of 5 μm and of 25 μm for natural and permed hair, respectively, with a broad distribution after the reductive treatment (Fig. 12). Taking the underlying strong degradation of hair structure into account, penetration of proteins in deeper sections of the cuticle can be assumed proven by the broad distribution of fluorescent areas in cross section images for protein treated permed hair (Figs. 9 and 10). By contrast, equivalent samples of untreated hair revealed only superficial deposits similar as shown in images of the fluorescein control on untreated hair (Fig. 11, part B).

4. Discussion

Hair fibers’ durability, water absorption capability, softness and flexibility as well as healthy appearance is a consequence of their underlying structure. Surrounded by the cuticle formed by 5–12 layers of overlapping scales acting as a barrier to chemicals, heat and mechanical damage, the cortex gives the fiber its high mechanical strength. Accounting for 70–95% of the total hair mass the latter comprises on the one hand long filaments formed by α-helical dimers of acidic type I and basic type II α-keratins arranged in the hierarchical structure of macro-fibrils [6]. On the other hand, globular KAP act as a disulfide crosslinker holding the cortical superstructure together.

In an approach to combine the inherent properties of these proteins for the optimization of keratin biomaterials the network structure of keratin hydrogels comprising α-keratins or mixtures of both keratin types was assessed. Though possessing a high cysteine content and smaller molecular mass, KAP did not act as a crosslinker and plasticizer but restricted the self-association of α-keratins [24]. As formation of aggregates and films on hair fiber surface was reported [7,12] these interactions between α-keratins and KAP may also affect the overall performance of keratin binding. The required separation of protein fractions was previously performed by isoelectric precipitation [24] or selective extraction with varying concentrations of alcohols [13]. As hydrolysis and fragmentation was found for products of the former procedure, extraction of KAP with the
addition of ethanol was chosen for separation in this study. By virtue of weakening the denaturation efficiency, presence of alcoholic hydroxyl groups in the extraction medium was observed to restrict extraction of micro-fibril proteins arranged in a tightly packed structure [1,13]. The employed two-step extraction enables selective fractionation of KAP and Ker proteins while keeping the advantages of the Shindai extraction representing a well-established extraction procedure with high yields, restricted protein hydrolysis as well as the lack of chemical modification of thiol groups.

Subsequent introduction of 6-MNA resulting in a structure being uncharged at physiological pH has been proven in its reactivity towards various thiol bearing mucosal tissues in different studies [15,25,26]. The negligible impact of this chemical modification on surface charge of the respective product was further intensified by addition of L-arginine being widely used to suppress protein aggregation [27]. Hair possessing an intact cuticle with its outermost layer, the epicuticle, grafted with fatty acids via thioester linkages, most abundant 18-methyleicosanoic acid (18-MEA), reveals a hydrophobic surface [28].
With an IEP of 3.67 virgin hair is moreover strongly negatively charged under the employed conditions for keratin binding [11].

Among strategies to enhance binding to natural hair by increasing reactivity and at the same time enhance hydrophobicity of proteins or reduce electrostatic repulsion a differentiated performance was observed for KAP and Ker. While the concept of preactivation of thiol groups was effective for both protein fractions, only Ker binding can be augmented by L-arginine. This behavior may be a consequence of α-keratins’ film forming characteristics [7] and its enhancement due to charge shielding properties of the added amino acid. Accumulation along the cuticle edges was previously reported for keratin [8,12] and moreover proteolipids composed of hydrolyzed wool keratin bearing an alkyl chain (C₈-C₁₈) as well as a quaternized adapter group [10]. Therefore, it can be assumed that this focus of accumulation may be independent of specific properties of the keratin derivative for untreated or aged hair. Opening and closing of cuticle scales favoring a mechanical

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**Fig. 11.** Hair treated with sodium fluorescein in a concentration of 4.0 μg/mL in 100 mM phosphate buffer pH 8.0 without treatment (A/B) and after perming of fibers (C/D) displayed as maximum intensity projections of hair surface (A, C) and cross section images (B, D).

**Fig. 12.** Distribution of sodium fluorescein displayed as normalized density in untreated (A) and permed hair (B) after incubation with sodium fluorescein in a concentration of 4.0 μg/mL in 100 mM phosphate buffer pH 8.0.
catching under conditions of binding studies were described as a possible mechanism behind this observation [12]. Villa et al. moreover attributed the deposition of feather keratin hydrolysates in cuticle junctions of natural and chemically treated fibers to sealing of cuticles improving color and shine of hair [8].

Further severe cuticle degradation was induced by perming known to cause stripping of the hydrophobic lipid layer [7]. TGA used as reducing agent in this study was proven to attack crosslinks between intermediate filaments and matrix proteins as well as within non-filamentous matrix being thus more effective than L-cysteine cleaving merely bonds between KAP components. In the process of reduction of disulfide bonds and subsequent reorganization a loss of disulfide bonds was reported due to oxidation of cysteinyl residues to cysteic acid as well as incomplete reoxidation to disulfides due to steric hindrance [29]. These profound alterations in hair structure were also reflected by the enhanced total water content of permed fibers [7]. Axial enfolding of fibers under the reductive perming conditions may be a consequence of extreme swelling at alkaline pH and subsequent dehydration as well as the possible release of proteinaceous material. Thus, a differential shrinkage of hair binding studies, KAP.

The enhanced penetrability of permed hair possibly occurs which is in agreement with SEM images of hair extracted with the Shindai method [6]. On this basis, the better performance of KAP derivatives in binding to the hydrophilic fiber surface stands in contrast to Ker possessing good film forming and self-association properties due to its fibrillar structure and higher molecular mass [24]. The enhanced penetrability of perm hair possibly allowing interactions with free thiol groups in deeper regions of the fiber may further contribute to augmented binding of smaller KAP.

Besides their use to detect distribution patterns in the course of binding studies, fluorescent and non-fluorescent dyes are widely employed to examine properties of virgin and chemically treated hair. Methylene blue staining and subsequent microphotospectrometry in combination with FT-Raman spectroscopy served to evaluate the impact of perming conditions on the penetration of TGA into the fiber. Due to the utilized set-up, however, only perming of white hair could be assessed [23]. The suitability of confocal laser scanning microscopy for the structural visualization of fluorescence signal in hair was demonstrated by Kelch et al. [22] finding nearly identical structures in cross sections of natural fibers when directly compared to scanning near field optical microscopy. This makes confocal microscopy as non-destructive method a promising tool for further studies also with smaller molecular mass substances being able to penetrate the cortex.

5. Conclusion

Based on encouraging results for enhanced binding of S-protected keratin to natural and bleached hair [12], a differentiated examination of factors that may further increase adhesion was performed in this study. Separated extraction of hair proteins as KAP and Ker fractions showed improbable Ker binding by addition of L-arginine. The introduction of an uncharged mercaptopropyridine derivative as leaving group by disulfide bond formation with cysteinyl residues in proteins was proven to strengthen accumulation on natural hair. Permanent waving treatment and consequent severe fiber degradation further demonstrated discrepancies in binding characteristics with a pronounced adhesion for KAP derivatives to the more hydrophilic surface. Alterations in hair structure and therefore higher penetrability after perming was underlined by control experiments with free fluorescent marker.

These findings may support the design of keratin protein containing systems that use the film forming and self-associating properties of α-keratins or the ability of KAP to accumulate on hydrophilic surfaces to restore the hair structure. In this context, further combination with quaternary residues or lipid components can be beneficial.

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CRediT authorship contribution statement

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Christina Leichner: Conceptualization, Methodology, Writing - review & editing.

Christian Steinbring: Methodology, Software, Investigation, Writing - review & editing, Visualization.

Andreas Bernkop-Schnürch: Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

None.

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