Short Communication

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Quantitative High-Performance Liquid Chromatography Analysis of Binding of Keratin-Derived Sequences to Hair Care Ingredients

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Various raw materials are used in the manufacture of cosmetics for hair repair. For instance, hair care ingredients comprising proteins, peptides, and free amino acids are used to enhance the texture of damaged hair resulting from repeated chemical treatments, such as perms and hair coloring. Hair repair methods that restore tensile strength have been shown to improve the mechanical properties of hair. However, the elucidation of the binding mechanisms of keratin proteins, the primary components of hair, to hair repair components is essential to understand the mechanism underlying hair repair. This is challenged by the lack of established methods for evaluating the binding of repair components to keratin proteins. We established an experimental system to evaluate the binding of hair repair components to hair proteins using two approaches. The first involved the synthesis of keratin-derived peptides with a high α-helical potential. The second approach involved detecting the above-mentioned interactions using high-performance liquid chromatography. This technique was successfully applied to the hair repair component S-carboxymethyl alanyl disulfide keratin, which was found to bind specifically to Type II-derived sequences.

Key words: hair, keratin, repair ingredient, disulfide bond, S-carboxymethyl alanyl disulfide keratin, high-performance liquid chromatography, α-helix, cosmetics, peptides, raw materials

1. Introduction

Hair covers a relatively large area of the head's surface and differs in color from the skin, and variations in hair shape can significantly affect an individual's appearance. Ensuring good hair quality and styling hair in multiple ways requires a proper understanding of the structure and function of hair to develop hair care strategies that are suited to distinct hair conditions. Most hair repair ingredients present in hair care products target keratin proteins, as approximately 85% of hair is composed of these proteins. Understanding the relationship between hair damage and structural changes in keratin proteins is of utmost importance, and establishing methods that identify the type and sequence regions within keratin proteins affected by repair components is crucial.

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Hair structure consists of the cuticle, cortex, and cell membrane complex. Proteins within the cortex are roughly classified into those with low cystine content and those with high cystine content.[1] While the former possess the same structure as intermediate filament (IF) proteins found in mammalian cells and are called IF proteins, the latter are called keratin-associated proteins (KAPs). IF proteins are typically classified into Type I proteins that are characterized by a molecular weight of 4.2–4.6 × 10^4 and many acidic side chains, and Type II proteins, with a molecular weight of 5.6–6.0 × 10^4 and several neutral or basic side chains. Stable dimers of these proteins are strongly involved in the structural organization of keratin.[2]

Hair care products contain a variety of proteins, peptides, and free amino acids that serve as hair repair ingredients aimed at enhancing hair health and beauty.[3] Several studies have demonstrated that low-molecular-weight peptides and amino acids diffuse into the cortex, reduce hair damage, and improve fiber mechanical properties.[4,5] The relationship between hydrolyzed keratin proteins and hair secondary structure has revealed that common peptide sequences between the hydrolyzed protein product and the amorphous segment of the IF of hair keratin result in effective enhancement of the tensile strength of hair.[6] However, the mechanisms by which such peptides and proteins interact with hair proteins to exert their functions are not well understood, and represent a bottleneck in the field of hair repair research, which requires a molecular structure-based approach. While western blotting, surface plasmon resonance, and fluorescence resonance energy transfer are typical methods used for protein interaction analysis, they are unsuitable for comprehensive screening methods to investigate proteins at the amino acid level. We devised a novel method that allows the binding properties of hair repair ingredients to be readily evaluated. This method relies on the preparation of keratin protein-derived peptides that are candidates for interactions with hair repair ingredients, and the quantitative analysis of the binding between these peptides and hair care ingredients by high-performance liquid chromatography (HPLC). The present study utilized S-carboxymethyl alanyl disulfide keratin (CMADK) as the repair ingredient.[7]

CMADKs not only exhibit high persistence on hair fibers but also inhibit cuticle lifting and improve fiber torsional stiffness rate upon deposition on the fiber surface. This has been attributed to their ability to penetrate the cuticle and form SS bonds within hair protein, and between the hair protein and cuticle via the SH/SS exchange reactions on being heated in the presence of water.[7] We synthesized a CMADK comprising all the hair proteins, including Type I proteins, Type II proteins, and KAPs. The average molecular weight of this CMADK was reduced by hydrolysis to facilitate its action on hair. We thus aimed to elucidate the binding regions in keratin that interact with the newly synthesized CMADK by developing an HPLC-based quantitative analysis method that utilizes partial keratin peptide sequences.

2. Materials and Methods

2.1. Extraction of sequences with high potential for binding repair components based on α-helical propensity

Keratin is a high-molecular-weight protein consisting of more than 400 amino acid residues. An exhaustive evaluation of CMADK binding to all regions of the protein is impractical because of the significant time investment required. It was therefore essential to theoretically narrow down the regions of keratin proteins toward which CMADK may demonstrate some degree of binding.

A total of 15 keratin proteins, including Type I: Ha1, Ha2, Ha3-I, Ha3-II, Ha4, Ha5, Ha6, Ha7, and Ha8; and Type II: Hb1, Hb2, Hb3, Hb4, Hb5, and Hb6, were evaluated. Elucidation of the mechanisms by which hair repair compounds exert their effects is challenged by the fact that the structures of the proteins they interact with have not been fully deciphered. We thus formulated the following two hypotheses to characterize the regions that potentially interact with CMADK. The first hypothesis postulates that compound binding to the α-helical region of keratin proteins stabilizes their structure, which was assessed by calculating the high α-helical propensity scores, H_n, for the amino acid sequences of different keratin proteins according to the Chou–Fasman method (a_i)[8] and the following equation: $H_n = \sum_{i=0}^{k} a_{(n+i)}$, where the subscripts n and k represent the corresponding residue numbers and the 21 residue motif ($n + 20$), respectively. The second hypothesis proposes that hair proteins and repair compounds interact via disulfide bonds. Attempts to verify this hypothesis involved the extraction of sequences consisting of the 21 residue motif, $(Xaa)_{21}$, which contains Cys residues capable of forming disulfide bonds.

2.2. Peptide design and preparation of extracted sequences

Keratin protein-derived peptides, such as Trp-βAla-$(Xaa)_{21}$-βAla-Arg, were designed by adding Trp, βAla, and Arg to the extracted region $(Xaa)_{21}$ to ensure sensitive detection by HPLC analysis, secondary structure interception, freedom control, and solubility enhancement. These peptides enabled verification of their binding to hair care components

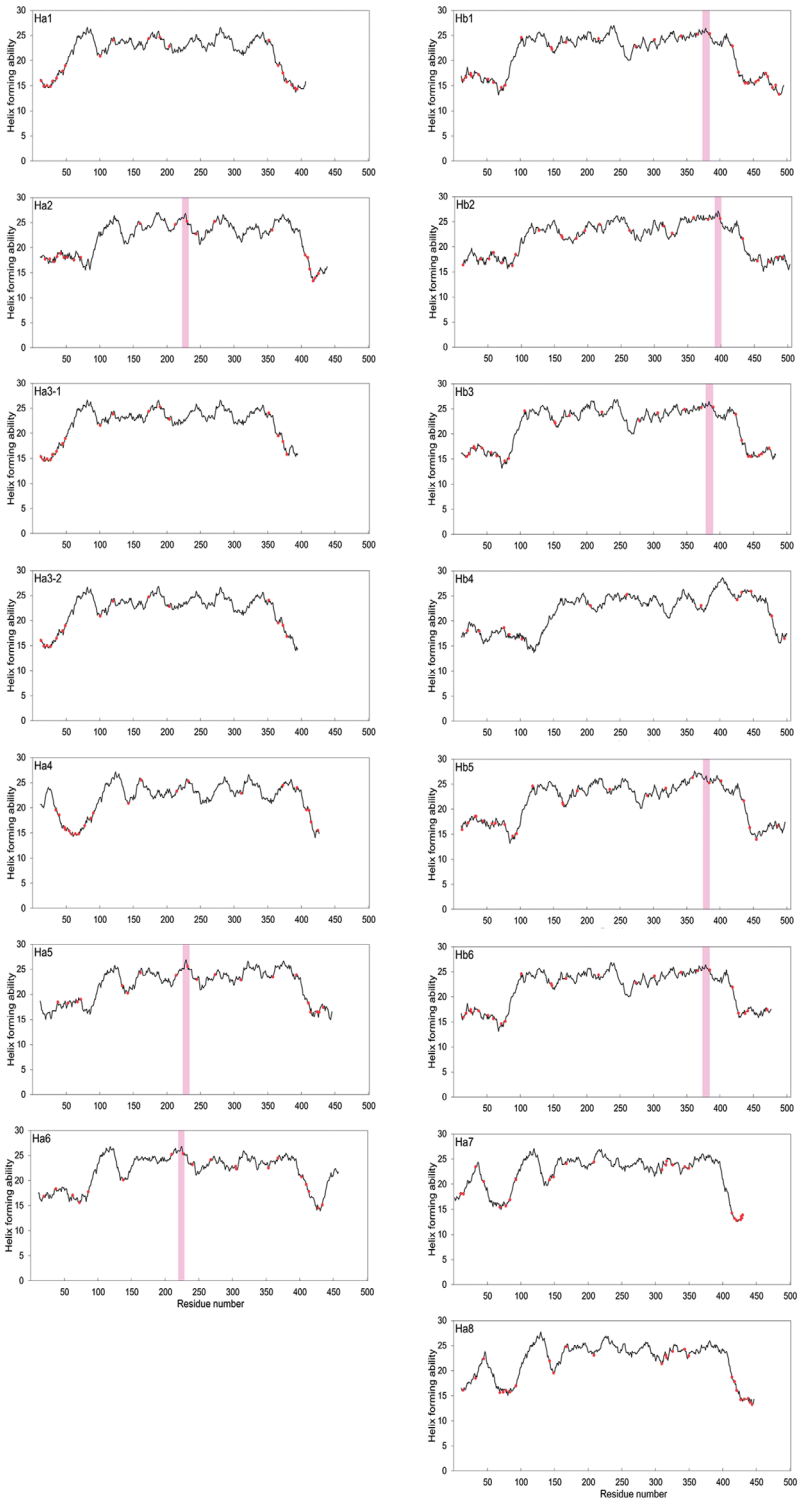

Fig. 1 Helix formation scores along the amino acid sequence of keratin proteins. The names of the different proteins are indicated within the respective graphs. The propensity scores for the 21-residue segments are denoted by a solid line. The highlighted regions indicate the sequences investigated in this study, which are listed in Fig. 2.

while preserving their secondary structure. All peptides were prepared via Fmoc solid-phase synthesis[9] and were dissolved in a 4:1 mixture of 50 mM phosphate buffer and acetonitrile to achieve a final peptide concentration of 0.4 mM.

2.3. Evaluation of binding of keratin protein-derived peptides to repair components by HPLC analysis

Solutions containing different amounts of Trp-βAla-$(Xaa)_{21}$-βAla-Arg and 0.1 equivalent of the repair component, CMADK, were prepared. Quantitative analyses were validated by performing HPLC analyses

Ha2(217-237),Ha6(214-234)	E A Q V E S L K E E L M C L K K N H E E E
Ha5(218-238)	E A Q V E S L K E E L L C L K K N H E E E
Hb1(368-388),Hb3(373-393),Hb6(368-388)	L A E L E G A L Q K A K Q D M A C L I R E
Hb2(385-405)	L E E A L Q K A K Q D M A C L L K E Y Q E
Hb5(369-389)	E A E Q Q G E A A L S D A R C K L A E L E

Fig. 2 Amino acid $(Xaa)_{21}$ sequences of the synthesized peptides, which were part of the Trp-βAla-$(Xaa)_{21}$-βAla-Arg general structure.

(L-5000; Hitachi High-Technologies Corporation) in peptide solutions with and without the addition of CMADK after 24 h. HPLC analyses were performed on an STR ODS-II (4.6 × 250 mm; SHIMADZU Corporation) column, using concentration gradients of 0.1% trifluoroacetic acid (TFA)/water and 0.1% TFA/acetonitrile at a flow rate of 1.0 mL/min. To improve the sensitivity of detection, quantification was performed by detecting the Trp absorption peak at 280 nm, which reduces the detection of multiple peaks that are usually evident when analysis is performed at 220 nm, the wavelength at which peptide bonds absorb light. The technique detects a decrease in residual peptide after CMADK binding. The presence or absence of CMADK binding was thus evaluated by comparing the percentage decrease in the peptide peak area before and after CMADK addition.

3. Results and Discussion

3.1. Screening of α-helical forming regions based on α-helical propensity scores

The α-helical propensities of the 21-residue segment for the entire length of each keratin protein, based on the Chou–Fasman method, are shown in Fig. 1. While the 50-residue regions at the N- and C-termini of all keratin proteins exhibited low α-helical potential, the 100–400 region demonstrated high α-helical potential. Further, among the regions with α-helical propensity scores of more than 26, 8 contained Cys residues (Fig. 1), which were presumed to be involved in binding to hair repair components.

3.2. Extraction of sequences with high potential for binding repair components

Of the proteins shown in Fig. 1, 8 regions containing the $(Xaa)_{21}$ amino acid sequence, which are likely to form α-helices owing to the presence of Cys residues that form SS bonds, were extracted (Fig. 2). Among these, Ha2 (217–237), Ha6 (214–234), and 3 other regions, including Hb1 (368–388), Hb3 (373–393), and Hb6 (368–388), shared identical sequences. This information was used to design and prepare 5 variants of Trp-βAla-$(Xaa)_{21}$-βAla-Arg, which were subjected to HPLC analysis.

3.3. Evaluation of the extent of binding of keratin-derived peptides to repair components by HPLC analysis

Figure 3 shows the peptide depletion results obtained from HPLC analysis of peptide solutions with and without CMADK. The results demonstrated clear differences in the peptide-derived peak areas of each peptide before and after the addition of CMADK, which was attributed to the absence and presence of CMADK binding, respectively. The percentage of depletion of each peptide was calculated from the difference in peptide peak area before and after CMADK addition (Fig. 4).

Type II-derived Hb2 (385–405) demonstrated the highest depletion percentage upon binding to CMADK. Decreased peak areas were also evident for other Type II-derived peptides, such as Hb1 (368–388), Hb3 (373–393), Hb6 (368–388), and Hb5 (369–389). These results suggest that these regions likely bind to CMADK. No reduction in peak areas was observed for Type II-derived peptides, such as Ha2 (217–237) and Ha6 (214–234), which are unlikely to bind to CMADK. This may be attributed to the non-formation of α-helical structures, which can be determined by evaluating the secondary structures of these peptides. A second possibility could be the involvement of factors other than the Cys-containing α-helix in binding to CMADK. For instance, the low binding affinity of Type I-derived peptides suggests that the basic protein does not participate in CMADK binding. This needs to be verified by further studies at the residue level that are based on the principles of protein science. The results obtained indicate that the high selectivity of recognition of hair repair components by specific peptide regions can be demonstrated using highly sensitive detection by HPLC and selective extraction of keratin protein-derived peptides. This study thus establishes a screening method for the identification of keratin regions that bind strongly to hair repair components by quantifying the decrease in peptide residues before and after CMADK addition by HPLC.

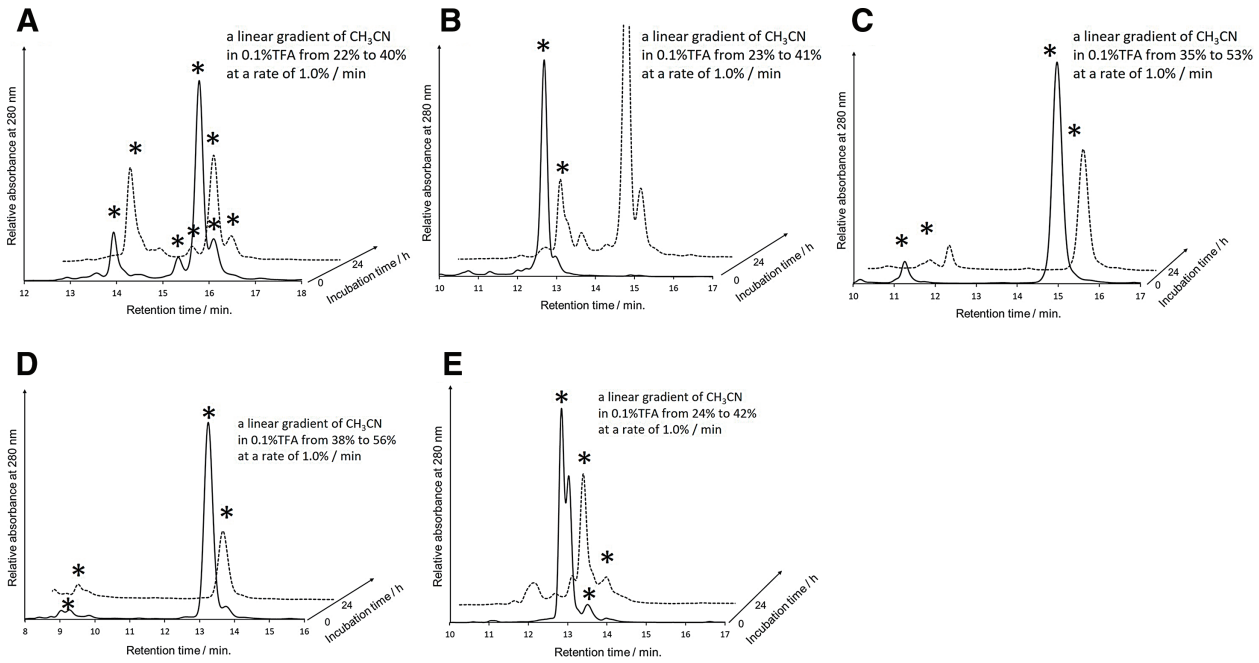

Fig. 3 HPLC profile of keratin-derived peptides alone (solid line) and in the presence of CMADK (dotted line). The keratin-derived peptides (asterisks) were eluted using a linear CH_3CN in 0.1% TFA gradient at a flow rate of 1.0%/min. (A) Ha2 (217–237), (B) Ha5 (218–238), (C) Hb1 (368–388), (D) Hb2 (385–405), and (E) Hb5 (369–389).

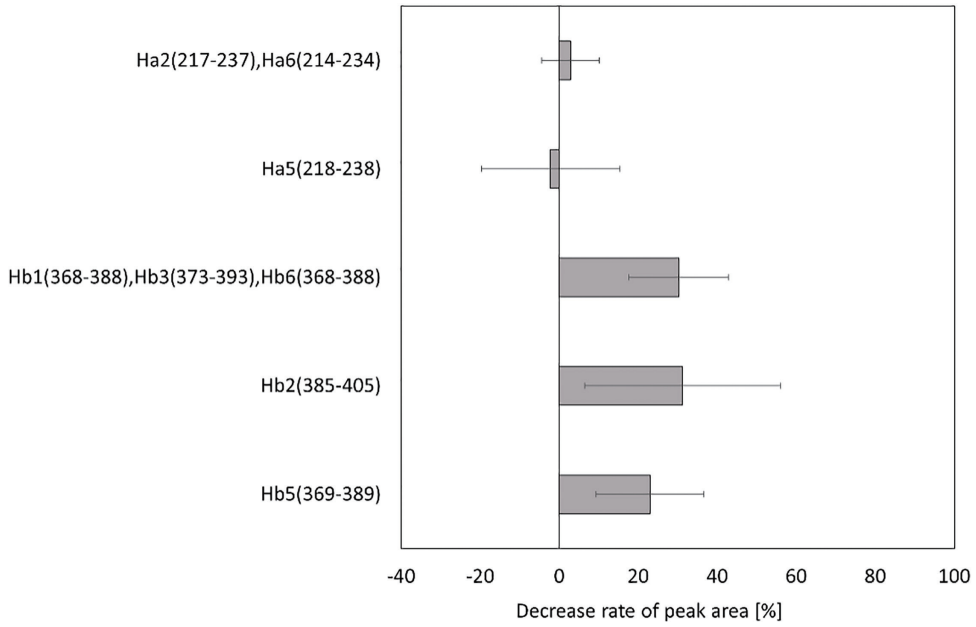

Fig. 4 Reduction ratio of keratin-derived peptide peak areas. Error bars represent the standard deviation of n = 6.

4. Conclusion

We demonstrate a novel and comprehensive method for the selective recognition of specific sites on keratin proteins by hair repair components. This was achieved by synthesizing keratin-derived peptides that could be detected with improved sensitivity. This pivotal method, established for the elucidation of the binding sites of hair repair components on hair proteins, is based on organic and analytical chemistry methods and utilizes protein science tools. The novel approach enabled us to overcome the challenges posed by structurally complex and diverse hair proteins that complicate

the identification of recognition sites. While the present study used the repair ingredient CMADK, the approach can be extended for the evaluation of other hair repair ingredients.

Conflicts of interest: The authors declare no conflicts of interest regarding this manuscript.

Abbreviations: CMADK, S-carboxymethyl alanyl disulfide keratin; HPLC, high-performance liquid chromatography; IF, intermediate filament; KAP, keratin-associated proteins; TFA, trifluoroacetic acid

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