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Proteomic tools for the investigation of human hair structural proteins and evidence of weakness sites on hair keratin coil segments

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ABSTRACT

Human hair is principally composed of hair keratins and keratin-associated proteins (KAPs) that form a complex network giving the hair its rigidity and mechanical properties. However, during their growth, hairs are subject to various treatments that can induce irreversible damage. For a better understanding of the human hair protein structures, proteomic mass spectrometry (MS)-based strategies could assist in characterizing numerous isoforms and posttranslational modifications of human hair fiber proteins. However, due to their physicochemical properties, characterization of human hair proteins using classical proteomic approaches is still a challenge. To address this issue, we have used two complementary approaches to analyze proteins from the human hair cortex. The multidimensional protein identification technology (MudPit) approach allowed identifying all keratins and the major KAPs present in the hair as well as posttranslational modifications in keratins such as cysteine trioxidation, lysine, and histidine methylation. Then two-dimensional gel electrophoresis coupled with MS (2-DE gel MS) allowed us to obtain the most complete 2-DE gel pattern of human hair proteins, revealing an unexpected heterogeneity of keratin structures. Analyses of these structures by differential peptide mapping have brought evidence of cleaved species in hair keratins and suggest a preferential breaking zone in α-helical segments.

Over the past decades, most research regarding keratin proteins has generally been focused on the chemical makeup of wool for textile and breeding purposes. However, during more recent years, investigations on human hair proteins have been meeting with a growing interest, especially in the field of cosmetic and dermatological sciences. A better understanding of this biological material and the structural organization of human hair protein may assist in product development and other potentially disease-related issues pertaining to hair and hair follicle proteins [1,2].

Outer hair is made of dead cells composed mainly of proteins that represent a range of 60–95% of total chemical composition. The other constituents are lipids, water, and metals whose levels may vary depending on the hair [3].

Hair fiber can be divided into three general components [4]: (i) an outer cuticle cell layer, (ii) an inner cortex, and (iii) a central medulla (in some cases). The cortex contains different cell types, and each cell contains 500–800 keratin intermediate filaments (KIFs)[2] that are the main proteins expressed in human hair. Human KIFs are composed of keratin proteins that can be sorted into two families: (i) the acidic type I keratins (K31–K38, containing 9 members) and (ii) the neutral basic type II keratins (K81–K86, containing 6 members) [5,6]. The complex interaction of type I and type II keratins results in the formation of heteropolymers [1,7–9].

These proteins are surrounded by an amorphous matrix of keratin-associated proteins (KAPs). The 26 KAP families represent

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[2] *Abbreviations used:* KIF, keratin intermediate filament; KAP, keratin-associated protein; HSP, high-sulfur protein; UHSP, ultra-high-sulfur protein; HGTP, high-glycine–tyrosine protein; UV, ultraviolet; PTM, posttranslational modification; MS, mass spectrometry; 2-DE gel, two-dimensional gel electrophoresis; p*I*, isoelectric point; MW, molecular weight; 2D, two-dimensional; MudPit, multidimensional protein identification technology; SCX, strong cation exchange; nanoLC–MS/MS, nano-liquid chromatography coupled to tandem MS; SDS, sodium dodecyl sulfate; DTT, DL-dithiothreitol; Na_2HPO_4, disodium hydrogen phosphate; NH_4HCO_3, ammonium bicarbonate; HPLC, high-performance liquid chromatography; CID, collision-induced dissociation; UPLC, ultra-performance liquid chromatography; TOF, time-of-flight.

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more than 100 KAPs with high-sequence homologies in each family.

These components form a strong compact and complex network of proteins bound together by intra- and intermolecular interactions such as disulfide linkages, hydrogen bonds, electrostatic salt bonds, and amide bonds [11,12]. This variety of internal bonds is essential to impart rigidity to the structure of the hair and make it resistant to environmental factors, such as ultraviolet (UV) exposure, pollutants, and weather, or chemical treatments [3].

Although the expression of type I and type II keratins and KAPs in the hair follicle is well established, their expression in mature hair, their posttranslational modifications (PTMs), and the way they can be altered in the hair shaft remain elusive. Their role in the shape and quality of hair, as well as the mechanisms involved, is not well identified [10,11].

Proteomics could assist in characterizing numerous isoforms and PTMs of human hair fiber proteins. Some challenges do remain regarding hair protein characterization using the general mass spectrometry (MS)-based strategies. Most of these challenges are due to the insolubility of hair proteins and the difficulty of extracting and solubilizing the proteins in solvents that are compatible with gel electrophoresis or liquid chromatography. Moreover, most hair proteins are keratins, and the issue of separating and detecting minor proteins (e.g., the KAPs) is still critical. Regarding this dynamic range issue, the high sequence homologies of human hair keratins (70–90%) and KAPs raise a major additional challenge that requires a high level of expertise to interpret generated mass data and identify the proteins [13–16]. The small number of unique peptides called proteotypic peptides constitutes an additional issue to address, to determine which particular members of these families are present in the sample.

So far, only a few proteomic approaches have been described in the way of analyzing human hair proteins. The first studies on keratin proteins were based on electrophoresis separation and were performed on wool keratins [17]. Similar studies were reported on human hair keratins that were identified by two-dimensional gel electrophoresis (2-DE gel) and Western blot analysis using antibodies specific to each family member [5,6,14]. The 2-DE gel of human hair keratins showed a pattern similar to the pattern of wool keratins, with a long train of proteins in the 62-kDa area between isoelectric point (p*I*) 5 and p*I* 7, which correspond to type II keratins, and a cluster of proteins at a lower p*I* and molecular weight (MW), which correspond to type I KIFs (p*I* 4–5, MW = 45 kDa). Beside this typical 2-DE gel pattern, few other spots appeared in the lower MW part of the gel.

However, most of the two-dimensional (2D) gel studies on the human hair shaft have demonstrated only the strong expression of some type I keratins (K31, K33a, K33b, K34, and K35) and type II keratins (K81, K82, K83, K85, and K86) without MS identifications [18]. As an alternative method for analyzing human hair proteins, Lee and coworkers suggested a multidimensional protein identification technology (MudPit). This approach involved separating a peptide complex mixture resulting from the digestion of total hair protein extract by strong cation exchange (SCX) chromatography followed by reversed phase nano-liquid chromatography coupled to tandem MS (nanoLC–MS/MS) [19,20]. This approach, which eliminated solubility problems and signal suppression due to the high number of generated peptides, allowed the authors to identify hair keratins, KAPs, and many proteins involved in the formation and structural organization of the hair shaft. In addition, Lee and coworkers showed evidence of posttranslational methylation, dimethylation, and trimethylation on hair proteins [21].

In the current study, we investigated an approach based on a combination of 2D gel and 2D LC followed by nanoLC–MS/MS to improve identification of human hair proteins.

Materials and methods

Protein extraction

All hair samples used in this study are constituted in the blending of untreated scalp hair from 3 individuals. Different extraction procedures were applied in accordance with the analytical method used. Before extraction, hair samples were delipidated by soaking fibers in ethanol and then in cyclohexane.

Protein extraction for 2D LC

Hair samples were extracted following the experimental procedure described by Lee and coworkers [21]. A short time after delipidation, proteins were extracted in a solution containing 2% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate (pH 7.8), and 20 mM DL-dithiothreitol (DTT) and were incubated overnight at 65 °C. After centrifugation, the insoluble material was submitted to a repeated extraction procedure (6 times) as described, finally giving two samples: an insoluble material and a soluble material.

Protein extraction for 2D gels

Proteins were extracted in a solution containing 7 M urea, 2 M thiourea, 50 mM Tris–HCl, 50 mM DTT, and 0.1% Triton X-100 for 18 h at 37 °C. The protein extract was collected via filtration and then alkylated with a solution of 1 M iodoacetamide and 3 M Tris–HCl at pH 8.4 for 10 min in the dark at room temperature. The solution was dialyzed with 3500-MWCO (molecular weight cutoff) dialysis cassettes (Pierce, Rockford, IL, USA) against water over a period of 48 h. The solution was then lyophilized and the freeze-dried sample was stored in a −80 °C freezer.

Separation and MS/MS analysis

Protein digestion and peptide separation by SCX chromatography

Both soluble and insoluble hair extracts were incubated for 1 h at 57 °C in 2% SDS, 20 mM DTT, and 50 mM disodium hydrogen phosphate (Na_2HPO_4) to reduce all cysteine groups. Reduced cysteines were then alkylated by adding 40 mM iodoacetamide for 1 h in the dark. Proteins were precipitated by adding 2.5 volumes of ethanol and rinsed twice with 70% ethanol to eliminate SDS, DTT, Na_2HPO_4, and iodoacetamide excess. Finally, proteins were resuspended in 100 mM ammonium bicarbonate (NH_4HCO_3) and 2 M urea and were digested overnight at 37 °C by adding modified porcine trypsin in a ratio 1 part of enzyme's weight for 20 parts of protein's weight.

Both tryptic peptide mixtures were fractionated with a Waters 625 LC System (Waters, Milford, MA, USA) using a PolySULFOETHYL A column (100 × 2.1 mm, 5 μm i.d., 300 Å pore size, PolyLC, Columbia, MD, USA) working at a flow rate of 200 μl/min. After samples were loaded, a 15-min isocratic run with 100% solvent A (5 mM KH_2PO_4 and 25% acetonitrile, pH 3.0) was performed. Peptides were eluted using a two-step gradient from (i) 0 to 25% of solvent B (5 mM KH_2PO_4, 350 mM KCl, and 25% acetonitrile, pH 3.0) in 30 min to (ii) 25–100% of solvent B in 20 min. Two-minute interval fractions were collected, concentrated by vacuum centrifugation, and desalted using ZipTip C18 Pipette Tips (Millipore, Bedford, MA, USA). A total of 35 fractions of each soluble and insoluble tryptic digestion material were collected for a second dimension of separation on reversed phase nanoLC coupled with MS.

Protein separation by 2-DE gel and in gel tryptic digestion

Protein sample (400 μg) was suspended in the rehydration buffer consisting of 7 M urea, 2 M thiourea, 2% Chaps, and 0.5% ampholytes (pH 3.0–11.0) and DeStreak rehydration solution (GE Healthcare, Uppsala, Sweden) and was incubated with IPG