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Isoelectric Focusing of Human Hair Keratins: Correlation with Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Patterns and Effect of Cosmetic Treatments

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ABSTRACT: A new isoelectric focusing (IEF) technique in polyacrylamide gels with 6*M* urea and 1.5% Nonidet P40 has been developed to characterize human hair samples. The phenotypes demonstrated with this procedure has been correlated with the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns described by other authors. The method described can be applied in the forensic science analysis of a single human hair. Using the same IEF technique we have studied the changes in electrophoretic patterns of cosmetically treated hair. The characteristics of the modifications observed and its utility in forensic science work are also discussed in this paper.

KEYWORDS: pathology and biology, hair, keratins, isoelectric focusing, noncarboxymethylated keratins

The ultimate purpose for forensic science hair comparison is to associate or not associate a hair with one particular person. There have been many attempts to develop techniques that can identify the origin of human hair samples. Conventional macroscopic and microscopic examination plays an important role in forensic hair comparison, but it has its difficulties [*1*]. In addition to this examination, some other tests have been performed. Measurement of trace element concentration in human hair has not proved successful. The ABO blood grouping is still regarded with some skepticism, and the characterization of hair by studying the isoenzimes and deoxyribonucleic acid (DNA) polymorphism in the follicle requires a plucked rather than a broken or shed hair with some of the outer root sheath present [*2*].

In the last few years, important advances have been made in the protein structure analysis of hair fiber, and a number of authors have suggested that analysis of hair proteins by electrophoretic procedures may be useful for discriminating between hairs from different species and for distinguishing hairs of different individuals within the same species. The electrophoretic systems extended over not only one-dimensional, but also two-dimensional electrophoresis and isoelectric focusing (IEF) [*3–12*].

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Isoelectric focusing in polyacryamide gels of noncarboxymethylated keratins has already proved its utility for species identification, and it could be used for detecting individual variation in a given population [*10*].

Heterogeneity and polymorphism of noncarboxymethylated keratins after sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been recently demonstrated [*11*,*12*].

In this paper, we report the correlation in the variation observed in both methods and the usefulness of the IEF followed by silver staining as a method for the analysis of such variation in forensic science cases.

Using the same IEF technique we have studied the changes in electrophoretic patterns of cosmetically treated hair. The characteristics of the modifications observed and its utility in forensic science work are also discussed.

Materials and Methods

Preparation of Hair Samples

Samples of scalp hair from 200 individuals and 20 families were studied. All donors were questioned about cosmetic manipulation of hair, and in order to analyze the variation in the keratin patterns, nontreated samples were selected.

On the other hand, additional hair samples of twelve individuals were collected with the aim of studying the changes in electrophoretic patterns of cosmetically treated hair. Each donor provided two samples, one before and one after cosmetic manipulation.

All the samples were washed successively with petroleum ether, ethanol, and water; air dried; and cut into small pieces.

Preparation of Soluble Proteins

Extraction of keratins was carried out according to Marshall and Gillespie [*8*] but without carboxymethylation [*10*].

Although the method was sometimes performed on a single hair (about 3 cm) extracted with 15 μL of extracting solution for 48 h at room temperature, usually proportionally higher amounts of hair and extracting solution were used.

The extracting solution was prepared by dissolving 0.09 g of TRIS (Tris(hydroxymethyl)aminoethane) (Sigma) and 7.2 g of urea (Merck) in 9.6 mL of water and then adding 120 mg of dithiothreitol (BioRad) immediately before use.

The extracting solution was briefly centrifuged and 5 μL of 0.1*M* dithiothreitol were added to 25 μL of supernatant at least 10 min before typing.

The sample was then ready to be run by isoelectric focusing.

Isoelectric Focusing

Isoelectric focusing was conducted using LKB systems Ultrophor, Multitemp, and Maxidrive 5000 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Polyacrylamide gels isoelectric focusing was carried out in 0.5-mm polyacrylamide gels at a gel concentration of $T = 4.8\%$ and cross-linking of $C = 3.2\%$. Sucrose (Merck) was added as a stabilizing agent at a total concentration of 12% (w/v).

The pH 2.5 to 8 range was used (prepared with equal parts of ampholine 2.5-4, pharmalyte 2.5-5, ampholine 4-6, and ampholine 6-8), and the total ampholine concentration was 2% (w/v). An urea concentration of 6*M* and a Nonidet P 40 concentration of 1.5% were added to the gel solution. Polymerization was carried out with 2% (v/v) riboflavin (Biorad) solution (20-mg/100-mL distilled water) under ultraviolet light (360 nm).

Samples were applied to Whatman 3 MM filter papers (0.5 by 0.5 cm) at a distance of 2 cm from the cathode. The electrode solutions were 1% (v/v) ethanolamine (Merck) for the cathode and 1*M* phosphoric acid (Merck) for the anode.

Focusing was carried out at 6-W constant power. A maximum voltage of 1500 V with unlimited current was used. Electrofocusing was carried out for 180 min, at a cooling temperature of 14°C.

SDS-PAGE

Electrophoresis was performed in a Protean II apparatus for vertical slab gels (BioRad). Discontinuous slab gels (1.5 by 110 by 230 mm) with homogeneous sample gels (T = 6%; C = 2.7%; pH 8.8) were prepared according to Laemmli [*13*]. Urea was added to the gelling solutions to a final concentration of 6*M* [*11*].

Gels were polymerized within 1 h at room temperature and prerun in electrophoresis buffer made up of 0.023*M* TRIS, 0.2*M* glycine, 0.2% SDS, pH 8.8 at 40 mA/gel for 1 h.

Hair extracts (10 to 30 μL) were loaded on the gels followed by electrophoresis at 40 mA/gel in the stacking gel and at 60 mA/gel in the sample gel until the dye track (bromophenol blue) has reached the lower end of the gel (3 to 4 h).

Staining

After isoelectric focusing and electrophoresis, the gels were stained with the silver staining method of Carracedo et al. [*14*].

Results and Discussion

Isoelectric focusing of noncarboxymethylated keratins has proved to be an useful method for identification purposes on forensic science casework since variations can be observed in old hairs as small as 2 cm [*10*]. Nevertheless, factors such as aging and sunlight exposure decrease the solubility of hairs although they do not modify its keratin electrophoretic patterns.

It has been suggested that urea [*15*] and detergents [*16*] could be useful to increase the solubility of keratins.

In this work, we have studied the optimal concentration of urea and NP 40 for IEF of noncarboxymethylated keratins. For this purpose, we have prepared linear gradients of both substances in IEF polyacrylamide gels (pH range 2.5 to 8) from 0 to 10*M* urea and 0 to 3% NP 40 (Fig. 1). From this experiment, we have proved as optimal conditions an urea concentration of 6*M* and a NP 40 concentration of 1.5%, and accordingly, we have carried out all the experiments with such conditions, obtaining always reproducible results.

Keratin heterogeneity by SDS-PAGE was analyzed by Gerhard [*11*]. This author could distinguish eight characteristic polypeptide patterns which were arbitrarily named K1 to K8. These phenotypes are characterized by the different numbers and patterns of major polypeptide bands in the range of 45 to 60 kDa. Using the same SDS-PAGE technique, Schimkat et al. [*12*] described four electrophoretic phenotypes named K1, K1m, K3, and K3m (Fig. 2). The authors propose that the four phenotypes observed are inherited in an autosomal dominant-recessive way, and that they are controlled by two independent loci K and m. The pedigrees indicate that the genotype of K1 is $*K^1/*K^1$, whereas the genotype of K3 is $*K^3/*K^1$ in the series of the studied families.

The genotype of non-m is *non/m/*non-m and the genotype of m is *m/*non-m. K3 is dominant and K1 is recessive. The modifying gene *m is dominant, while *non-m can be called recessive.

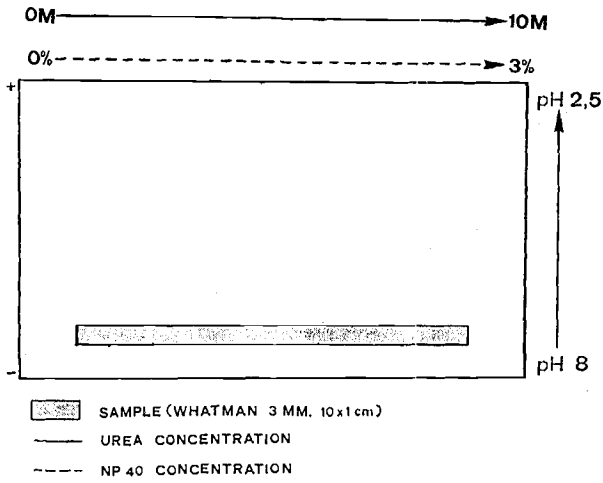

FIG. 1—*Linear gradients of urea and Nonidet P40.*

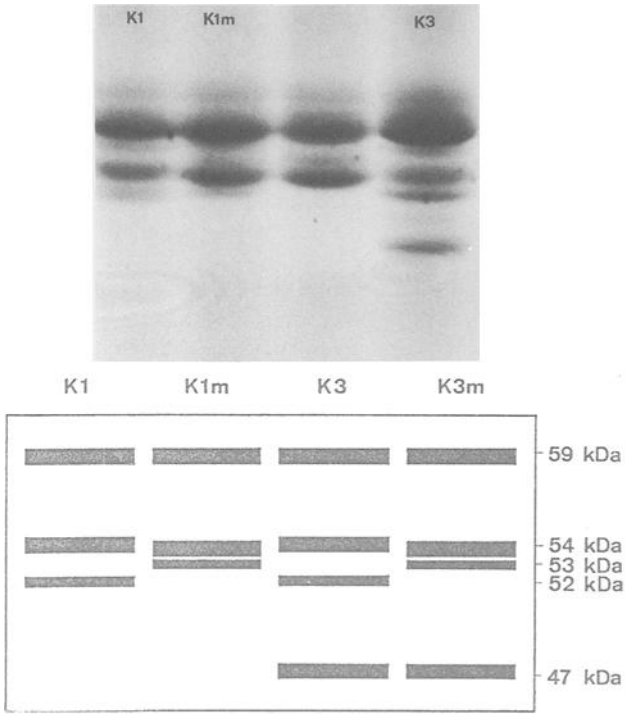

FIG. 2—*SDS-PAGE patterns of human hair keratins:* (a) *homogeneous polyacrylamide gel* (T = 6%, C = 2.7%, *pH 8.8) with 6M urea and* (b) *schematic representation of the patterns.*

Taking into account the results of the different authors, we believe that a new standardized nomenclature would be needed; even more so, if we take into consideration that the nomenclature of the hair protein proposed by Schimkat et al. [*12*] is unfortunate in the sense that it is easy to confuse with the classical KM (immunoglobulin light chain) polymorphism.

Correlation of keratin SDS phenotypes and IEF patterns is shown in Fig. 3, the patterns being designated as above.

A clear identification of all keratin phenotypes by IEF is possible, even with broad pH ranges such as pH 2.5 to 8. Isoelectric focusing has a number of advantages compared with SDS-PAGE, the most important ones being the high sensitivity, the accuracy for distinguishing some of the patterns, and the concentration effect of IEF which permits phenotyping of old hairs (even many years old) as small as 2 cm. Furthermore, cosmetic treatments can be directly detected by IEF.

Although it has been reported by some authors [*9,17*] that cosmetic treatments such as bleaching and permanent waving have no effect on the electrophoretic pattern of hair proteins, recent investigations [*8,18–20*] on the effects of these treatments by one- and two-dimensional electrophoretic procedures have shown remarkable differences in the patterns before and after treatment.

Changes in the keratin pattern of cosmetically treated hairs have been observed by using IEF on noncarboxymethylated keratins in the presence of 6*M* urea and 1.5% NP 40.

In the case of permanent-waved hairs, no additional bands were observed, but quantitative changes in the bands were detected in some cases. These quantitative changes consist of a decrease in the intensity of some bands after permanent waving, which can

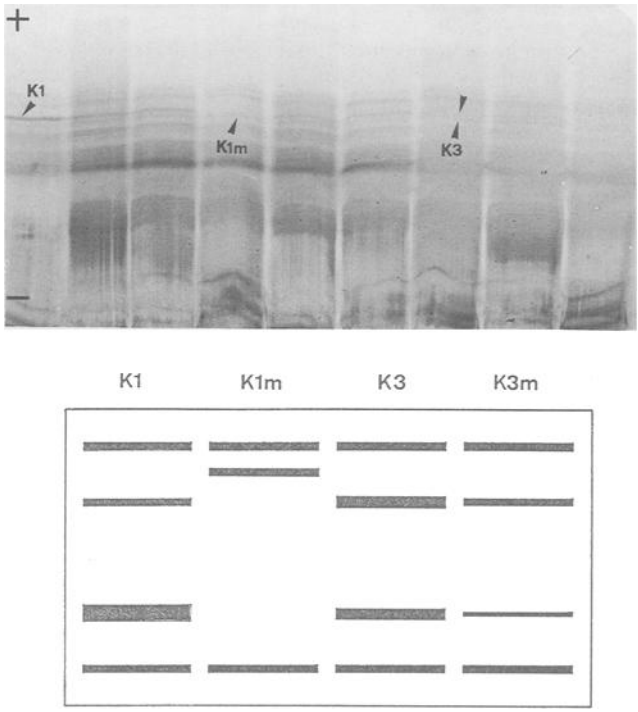

FIG. 3—*IEF patterns of human hair keratins:* (a) *polyacrylamide IEF gel* (T = 4.65%, C = 3.2%, *pH 2.5-8) with* 6M *urea and 1.5% Nonidet P40 and* (b) *schematic representation of the patterns.*

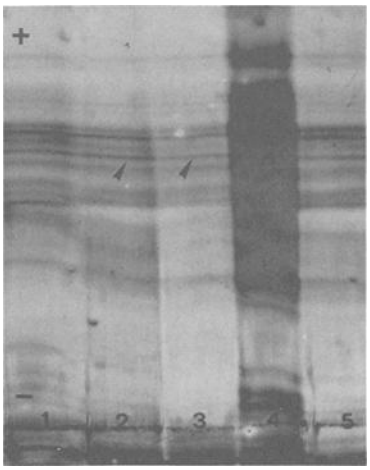

FIG. 4—*IEF patterns of cosmetically-treated hair (Lanes 1, 2, and 5: control hair; Lane 3: permanent-waved hair; and Lane 4: bleached hair).*

be quantified with the aid of a densitometer. These quantitative changes do not impede clear typing.

In the case of bleached hairs, the electrophoretic pattern was clearly modified with new strongly stained bands in the anodic area of the gel (Fig. 4). This modification is characteristic of this type of treatment and could be useful for individual identification. Although this cosmetic treatment does not allow a correct reading of the phenotypes, the characteristic pattern gives an additional data for identification purposes.

Miyake and Seta [*21*] have recently reported that no bands corresponding to high sulfur keratins are observed with the method of Gerhard [*11*]. This suggests that the pattern observed might be the combination of low sulfur proteins (LSP) with high sulfur proteins (HSP), which have a higher content of cystein residue.

To confirm whether the SDS-PAGE bands come from HSP or not, the IEF pattern is not very clarifying, since the pH range (4 to 4.5) of the variation observed is consistent with LSP or HSP.

Though all these problems are not solved and additional research is needed, the reproducibility in observing major phenotypes, at least with this IEF method, demonstrated the potential in applying this method to forensic science hair comparison.

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