Analysis of α-Keratins in the Horns of Rhinoceros and Buffalo by Non-Native Capillary Isoelectric Focusing

T.-L. Huang[1]* / P. Yu[2]

[1]Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92634, USA
[2]Beckman Instruments (Taiwan) Inc., 8F, 216 Tun Hwa Rd., Sec. 2., Taipei 106, Taiwan, ROC

Key Words

Capillary isoelectric focusing
Horn of rhinoceros and buffalo
α-Keratins
Urea

Summary

A capillary isoelectric focusing (CIEF) method under denaturing condition has been developed for separation of α-keratins, highly cross-linked biomacromolecules, in horns of rhinoceros and buffalo. The α-keratins were denatured in 8 M urea and separated in the presence of ampholytes with applicable pH ranges of 3–7 or 3–10. In the preliminary results, it was demonstrated that the α-keratins in the horns of buffalo or rhinoceros might have their own unique isoelectric focusing profiles. The analyses of more samples from different rhinoceros and buffalo are presently underway, with the final goal to establish a fast and convenient method for identification of the source (buffalo or rhinoceros) of an unknown powdered sample.

Introduction

The horn of rhinoceros in powder form has long been used in folk medicine as an antipyretic in Asia. Due to the amazing power of reducing fever as well as the myths that the horns contain an aphrodisiac [1], rhinoceroses have been killed extensively, although most rhinoceroses are now protected by law. The price of rhinoceros horn in the black market is 10–100 times as expensive as an equivalent weight of gold, encouraging the illegal slaughter of this endangered animal. People suspected of selling the powdered rhinoceros horns often claim that the powder is from the buffalo horns. Thus, identification of rhinoceros horns has become an urgent task for convicting and prosecuting people suspected of selling and smuggling horns from this endangered animal.

Owing to the merit of on-column detection and the capability of attaining high-resolution separation comparable to the conventional isoelectric focusing, capillary isoelectric focusing (CIEF) method has recently attracted considerable attention as a fast analytical tool for profiling of biomolecules [2–3]. The use of narrow diameter (25–50 μm ID) capillary with CIEF further offers the advantage of minute sample requirement (ng). In the present work, a non-native CIEF method was established to analyze α-keratins, the biological constitutes in the horns of rhinoceros and buffalo. Capillary isoelectric focusing of α-keratins was studied for the purpose of showing the applicability of CIEF to identify the source of a suspected powder of the rhinoceros horn.

Experimental

Materials

Powdered forms of horns of rhinoceros and water buffalo were obtained from the Investigation Bureau, Ministry of Legal Affairs, Taipei, Taiwan. The samples were dissolved in 0.1 M NaOH at a concentration of 5 mg mL^{-1}. The sample solutions were either analyzed immediately or stored frozen at –30 °C until required. Bare fused silica capillaries of 50 μm ID were obtained from Polymicro Technologies, Inc. (Phoenix, AZ, USA). Servalyts 3–7 was purchased from Serva (Crescent Chemical Company, Hauppauge, NY, USA). eCAP cIEF 3–10 Ampholyte, polymeric solution (with a trade name of eCAP cIEF gel), and neutral coated capillaries of 50 μm ID from eCAP cIEF 3–10 kits (Beckman, Fullerton, CA, USA) were used in this study. The coating inside the capillary wall consists of covalently bonded layers [4], which can effectively reduce the electroosmotic flow (EOF) in the capillary to $0.05–0.5 \times 10^{-4}$ $cm^2/V–s$ at pH 2–10. EOF has been shown to distort the linearity of pH gradient in a non-coated capillary with capillary isoelec-

0009-5893/97/10 437-03 $ 03.00/0

tric focusing (CIEF) [5]. All other chemicals used in this study were from Sigma Chemical (St. Louis, MO, USA).

Non-Native Capillary Isoelectric Focusing

A P/ACE 2210 capillary electrophoresis instrument controlled by System Gold software (Beckman, Fullerton, CA, USA) was used. The total capillary length was 27 cm, with a separation length (from injection to window) of 20 cm. The separations were carried out under normal polarity (inlet is the anode) with detection at 280 nm. Before uses, the bare fused silica capillaries were pretreated with 1 N NaOH for 5 min, followed by a 5 min rinse with deionized water. The sample was dissolved in an ampholyte solution before injection. The ampholyte solution consisted of 3 % eCAP cIEF Ampholyte 3–10 (or 1.6 % Servalyte 3–7) and 8 M urea in the eCAP cIEF gel. The capillary was prepared for a CIEF run as follows. The capillary was first filled with a mixture of ampholyte solution and samples, by applying the high-pressure rinse mode (20 psi) for 1.5 min. A field strength of 300 V/cm (2 min) was first applied to focus the ampholytes and samples. At the end of 2 min, the field strength was gradually increased to 500 V/cm in one min, to further focus the sample zones. Once reaching 500 V/cm, the system was programmed to apply a low-pressure rinse mode (0.5 psi) to mobilize the focused zones past window for detection, while applying the high voltage simultaneously to maintain the pH gradient in the presence of distorting effects of hydrodynamically induced laminar flow [5]. The catholyte was 20 mM NaOH solution with 8 M urea. With the uses of eCAP cIEF 3–10 Ampholyte, the anolyte was 91 mM phosphoric acid with 8 M urea in the cIEF gel solution; while with the uses of Servalyt 3–7, the concentration of phosphoric acid was optimized at 50 mM. Between runs, the column was rinsed with 10 mM phosphoric acid for 1 min, followed by deionized water for 2 min.

Results and Discussion

As shown in Figure 1, the horn of a rhinoceros lacks a bony core and is composed entirely of α-keratins [1]. The keratins are fibrous, insoluble proteins of animals derived from ectodermal (skin) cells [6]. There are two classes of keratins: α- and β-keratins. Compared to the β-keratins, the α-keratins are relatively rich in cystine residues and thus contain many disulfide cross bridges; in addition, they contain most of the common amino acids [7, 8]. The α-keratins include the hard, brittle proteins of horns and nails, which has a very high content of cystine (up to 22 %), as well as the softer, more flexible keratins of skin, hair, and wool, which contain about 10–14 % cystine [9]. The frequent primary valence cross-links (disulfide bonds) and secondary valence cross-links (hydrogen bonds) between neighboring polypeptide chains stabilize the keratin structure and make it difficult to dissolve the α-keratins in aqueous or organic solvents.

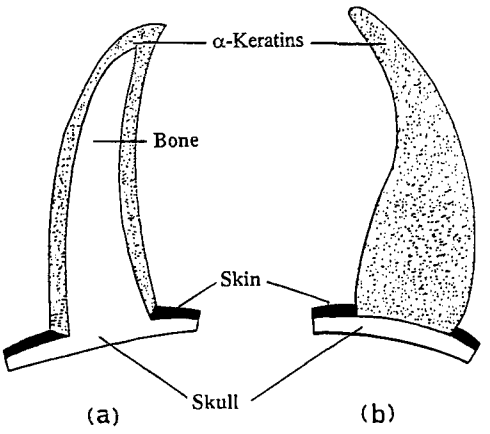

Figure 1

Schematic diagrams of horns of (a) buffalo and (b) rhinoceros. Compared to buffalo, the horn of rhinoceros lacks a bony core and is composed of solid α-keratins.

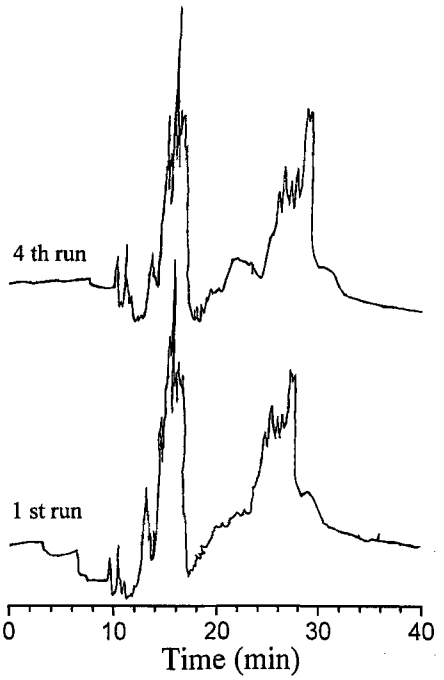

Figure 2

CIEF electropherograms of the α-keratins in rhinoceros horn. The separation conditions, amount of sample injection and the concentration of sample in the ampholyte solution were the same as Figure 3.

At the beginning of study, a native (without the use of denaturant) capillary isoelectric focusing method was used to generated the electrophoretic profiles of the α-keratins in the horns of rhinoceros and buffalo. However, it was not possible to obtain reproducible separation patterns, since α-keratins tended to aggregate through the inter- and intra-molecular disulfide bonds and hydrogen bonding interactions during the isoelectric focusing (IEF) course. Aggregation of keratins also caused protein precipitation, resulting in capillary clogging and irreproducibility of migration time. To solubi-

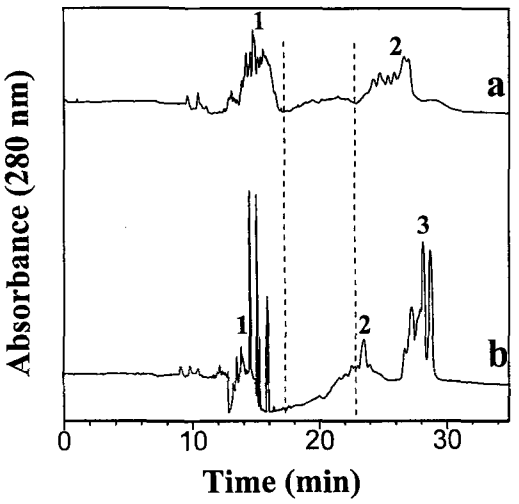

Figure 3
Comparison of CIEF electropherograms of the α-keratins in horns of (a) rhinoceros and (b) buffalo using the eCAP cIEF ampholyte 3–10. The sample was dissolved at a concentration of 125 μg mL^{-1} in an ampholyte solution consisting of 3 % eCAP cIEF ampholyte 3–10 and 8 M urea in the eCAP cIEF gel. The amount of sample injection was 158 ng. The separations were performed at 20 °C in a bare fused silica capillary.

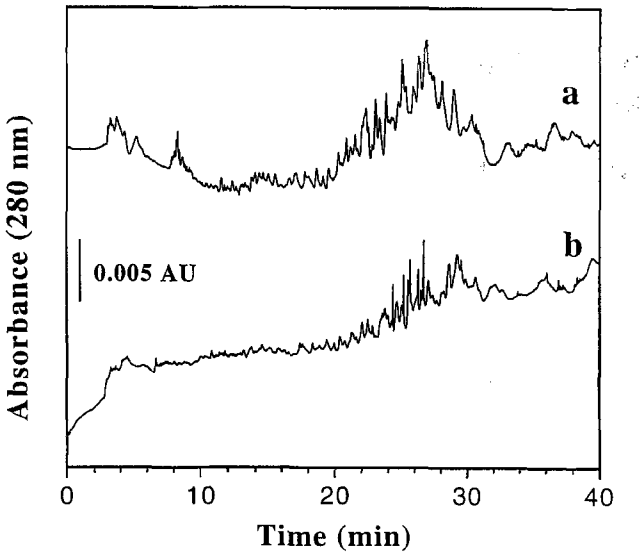

Figure 4
Comparison of CIEF electropherograms of the α-keratins in horns of (a) rhinoceros and (b) buffalo using the Servalyt 3–7 as ampholyte. The amount of sample injection and the concentration of sample in an ampholyte solution, a mixture of 1.6 % Servalyt 3–7 with 8 M urea in the eCAP cIEF gel, were the same as Figure 2. The separations were performed at 30 °C in a neutral coated capillary.

lize the α-keratins, urea was used as denaturant to disaggregate the keratin-keratin complexes. It was found that the use of 8 M urea could successfully reduce protein precipitation and improve reproducibility in separation patterns. See Figure 2. Thus, a denaturing (with 8 M urea) IEF method was then developed for generating the IEF profiles of keratins.

With the uses of eCAP cIEF Ampholyte 3–10, electropherograms of α-keratins in horns of the rhinoceros and buffalo show various peaks corresponding to different isoelectric points (pI's) in the pH range of 3–10 (Figure 3). At least two major peak domains were observed with the rhinoceros horn. Domain 1 had a migration time of 12–16 min, and domain 2 had a migration time of 24–28 min. In addition, there was a hump observed between peak domains 1 and 2, as indicated by the area between two vertical dash lines. In contrast, at least three major peak domains were observed with the buffalo horn. Domain 1 had a migration time of 13–16 min, and domains 2 and 3 had migration times of 21–25 min and 26–29 min, respectively. The hump shown in Figure 3(a), between peak domains 1 and 2, was investigated by using an ampholyte with applicable IEF range of pH's 3–7, to further increase the separation resolution at the particular pH range. The result was shown in Figure 3, together with the result of a buffalo horn using the same ampholyte. Comparing Figures 4 (a) and (b), there were significant quantitative differences in separation patterns between the keratins in the horns of buffalo and rhinoceros, presumably due to the differences in compositions and relative contents of the keratins (with pI's of about 3–5) from the two difference sources.

Acknowledgments

The authors gratefully acknowledge Chi-Sun Chao (Ministry of Legal Affairs, Taipei, Taiwan, ROC) for kindly providing the powdered samples of the horns of rhinoceros and water buffalo. We thank Beckman Publication Review Committee for helpful suggestion. We also thank Laurel Crump and Chialing Wu for proofreading the manuscript.

References

[1] "The Encycloppedia Americana" vol. 14, Grolier Inc., Danbury, 1993, p. 384.
[2] *F. Kilar, S. Hjertén*, J. Chromatogr. **638**, 269 (1993).
[3] *A.-K. Englund, P. Lundahl, K. Elenbring, C. Ericson, S. Hjertén*, J. Chromatogr. A **711**, 217 (1995).
[4] *T.-L. Huang, P. C. H. Shieh, N. Cooke*, J. Chromatogr. A **685**, 313 (1994).
[5] *T.-L. Huang, P. C. H. Shieh, N. Cooke*, Chromatographia **39**, 543 (1994).
[6] *J. H. Bradbury*, Adv. Protein Chem. **27**, 111 (1973).
[7] *W. H. Ward, H. P. Lundgren*, Adv. Protein Chem. **9**, 243 (1954).
[8] *W. G. Crewther, R. D. B. Fraser, F. G. Lennox, H. Lindley*, Adv. Protein Chem. **20**, 191 (1965).
[9] *H. R. Mahler, E. H. Cordes*, "Biological Chemistry", 2nd edn, Harper & Row, Publishers, Inc., New York, 1971, p. 126.

Received: Apr 29, 1996
Revised manuscripts
received: Dec 13, 1996 and Mar 6, 1997
Accepted: May 14, 1997