Short Communication

Presence of Citrulline in the Membranous Proteins of Stratum Corneum of Newborn Rat and Cow Snout

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Citrulline is known as an intermediate metabolite in the urea cycle and is not found usually as a component of protein. However this amino acid has been identified as a constituent of proteins from the inner root sheath of hair follicles, medulla of hair and quills,^{1,2)} and human myelin.³⁾ The presence of citrulline in the protein of horny cell membrane of the epidermis has not been previously reported. This paper identifies citrulline in the proteins of horny cell membranes in the epidermis of the newborn rat and cow snout. This was confirmed by a colorimetric determination of citrulline in the polypeptides released from the horny cell membrane by tryptic digestion. Exhaustive proteolytic digestion of these polypeptides showed that citrulline was covalently bound by peptide linkage to the membranous proteins.

The horny cell membranes of the epidermis of newborn rats (2~3 days old), cow snout and human plantar callus were prepared by the "alkali method" according to Matoltsy and Matoltsy⁴⁾ with slight modification. The membranes from the epidermis of newborn rat were further purified by discontinuous sucrose density gradient ultracentrifugation.⁵⁾ The membranes were obtained at interface d 1.0/ d 1.16. The final pellets of each sample were checked by phase-contrast and electron microscopy, washed with pure water and lyophilized.

For proteolytic digestion, membrane preparations were suspended in Tris buffer (0.1 M

Tris-5 mM CaCl_2 , pH 8.0) and heated in a boiling water bath for 5 min. Then the suspensions were digested at 37°C by the sequential addition of Pronase P, carboxypeptidase A, prolidase and aminopeptidase essentially as previously described.^{6,7)}

The identification and determination of citrulline in the digests of the membranes was carried out as follows. The supernate of the digests was applied to a column of Bio-Gel P-2 (2.1 $\times$ 90 cm). The column had previously been calibrated with authentic citrulline. The citrulline fractions were collected and analyzed by a Hitachi KLA-3B amino acid analyzer with a custom 2612 resin column (0.9 $\times$ 52 cm). The elution was carried out with 0.25 N lithium citrate buffer for the biological materials by the method of Murayama and Shindo⁸⁾ with the following modifications; pH 2.70 buffer was used instead of pH 2.90 buffer for the first buffer, and eluted at 35°C. The proteolytic digests of the membranes from newborn rat and cow snout exhibited a peak that eluted at about 250 min. The peak was located between alanine (229 min) and valine-cystine (280 min). When authentic citrulline was added to the proteolytic digests of the membranes, the elution pattern showed augmentation only in the peak eluting at 250 min. Authentic homocitrulline appeared at 280 min and was completely separated from citrulline. When acid hydrolysates (6 N HCl 110°C, 17 hr) of the membranes were analyzed, similar results were obtained. For identification of citrulline, the citrulline fractions of the digests, freed from basic amino acids on a Bio-Gel P-2, were collected and hydrolyzed with 0.2 N sodium hydroxide at 110°C for 24 hr.⁹⁾ They were then applied to a 52 cm column of the analyzer and eluted with 0.2 N sodium citrate, pH 4.25 as the initial buffer for 30 min and 0.48 N sodium citrate, pH 6.1 as the second buffer at 35°C. Only ornithine (156 min) which is derived from citrulline by alkaline hydrolysis was detected. No lysine (167 min) was observed. This observation excludes the presence of homocitrulline which is formed by reaction of cyanate with the ϵ - NH_2 groups of

FIG. 1. Gel Filtration of Proteolytic Digest of Horny Cell Membranes of Newborn Rat on Bio-Gel P-2.

The column (2.1×43 cm) was equilibrated and eluted with 50 mM ammonium bicarbonate. (A) Tryptic digest; (B) The excluded tryptic polypeptide which was exhaustively digested with proteases.

○—○, citrulline; ●—●, protein (Lowry); ○---○, authentic citrulline.

lysine residues.

Citrulline was detected in the horny cell membranes of the epidermis of the newborn rat ($140 \mu\text{mol}$ per g of protein) and cow snout ($190 \mu\text{mol}$ per g of protein) but not detected in the membranes of human plantar callus. A low level of citrulline was found in the alkali soluble proteins of the epidermis from the newborn rat ($12.5 \mu\text{mol}$ per g of protein) and cow snout ($7.2 \mu\text{mol}$ per g of protein).

Since citrulline loosely associated with the proteins might not have been removed during the preparation of the membranes, it was necessary to provide evidence that citrulline was bonded covalently as a peptide bond in

the proteins. This was confirmed by the proteolytic release of citrulline from the protein described as follows. The horny cell membranes were digested with trypsin (1:100 w/w) in the Tris buffer at 37°C for 24 hr. The supernate of the digests was fractionated by gel chromatography on a column of Bio-Gel P-2 (2.1×43 cm). Typical elution profile shows in Fig. 1A. Only the color reaction of citrulline with diacetyl monoxime¹⁰⁾ was observed in the polypeptide fractions which appeared in the vicinity of the void volume. After exhaustive proteolytic digestion the digests of these excluded polypeptide fractions of each column were again chromatographed on the column of Bio-Gel P-2. The column had previously been calibrated with authentic citrulline. Representative results are shown in Fig. 1B. The proteolytic digests of the polypeptides exhibited a peak that was eluted as a retarded fraction. This peak coincided with that of authentic citrulline. When this peak was concentrated and applied on a column of amino acid analyzer as described above, citrulline was identified.

The results presented in this paper show that citrulline is present in a protein fraction isolated from the horny cell membranes of the epidermis of newborn rat and cow snout. The citrulline residues of the membranous proteins could have been derived from the arginine residues by post-translational processing of the proteins in the late stages of epidermal differentiation. Further studies are needed to determine the biochemical role of the presence of citrulline residues in epidermal differentiation including the formation of horny cell membranes. The presence and the properties of the specific enzyme in the epidermis which catalyzes the conversion of arginine residues to citrulline residues will be published in the following paper from this laboratory.

REFERENCES

- 1) P.M. Steinert, H.W.J. Harding and G.E. Rogers, *Biochim. Biophys. Acta*, **175**, 1 (1969).
- 2) H.W.J. Harding and G.E. Rogers, *Biochemistry*,

- 10, 624 (1971).
- 3) P. R. Finch, D. D. Wood and M. A. Moscarello, *FEBS Letters*, **15**, 145 (1971).
- 4) A. G. Matoltsy and M. N. Matoltsy, *J. Invest. Dermatol.*, **46**, 127 (1966).
- 5) P. Emmelot, C. T. Bos, E. L. Benedetti and D. H. Rumke, *Biochim. Biophys. Acta*, **90**, 126 (1964).
- 6) H.G. Williams-Ashman, A.G. Notides, S.S. Pabalan and L. Lorand, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2322 (1972).
- 7) K. Sugawara, "Biochemistry of Cutaneous epidermal differentiation," ed. by M. Seiji and I. A. Bernstein, University of Tokyo Press, Tokyo (1977) pp. 387.
- 8) K. Murayama and N. Shindo, *J. Chromatogr.*, **143**, 137 (1977).
- 9) G. R. Stark and D. G. Smyth, *J. Biol. Chem.*, **238**, 214 (1963).
- 10) G. Guthöhrlein and J. Knappe, *Anal. Biochem.*, **26**, 188 (1968).
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