Short Communication

Presence and Localization of
Citrulline in the Protein
of Rabbit Skeletal MuscleJunichi NISHIMAKI,* Kiyoshi SUGAWARA
and Takeshi OUCHI***Laboratory of Biochemistry,
Department of Agricultural Chemistry,
Ibaraki University, Ami-machi,
Inashiki-gun, Ibaraki 300-03, Japan*

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In a previous paper, we reported the presence of peptidylarginine deiminase which catalyzes the formation of citrullyl residues from arginyl residues in rabbit skeletal muscle.¹⁾ However, the presence of citrulline in the protein of muscle has not previously been reported. To study the biochemical function of this enzyme, we thus attempted to detect the reaction product of this enzyme in rabbit skeletal muscle. This paper reports the presence of citrulline in the protein of rabbit skeletal muscle and its localization in the membranes.

Fresh skeletal muscle of rabbit (2.5 kg) was homogenized with an Ultra Turrax in 5 mM Tris-HCl (pH 7.3) containing 0.5 mM EDTA (buffer A) at 0°C. The homogenate was filtered through double layers of cheese-cloth. The filtrate was centrifuged at $10,000 \times g$ for 10 min at 0°C, the sediment (S_1) was washed three times with cold 5% trichloroacetic acid (w/v), and then delipidated with chloroform-methanol-water (1:2:0.28, v/v).²⁾ The delipidated S_1 was used as the whole muscle preparation. The preparation of light surface membranes by the low-salt sucrose procedure was carried out according to the method of Barchi *et al.*³⁾ with slight modification as follows. S_1 was homogenized with a Ten Broeck

tissue grinder in buffer A and the homogenate was centrifuged at $10,000 \times g$ for 10 min at 0°C. The pellet was resuspended in buffer A and layered on a 10:30% (w/w) discontinuous sucrose density gradient and centrifuged to equilibrium (at $100,000 \times g$ for 16 hr). The membranes were obtained at the 10:30% interface and the pellet obtained at the bottom was designated as the nonmembrane fraction. Actin, myosin and connectin were prepared from the skeletal muscle of rabbit by the methods of Mommaerts,⁴⁾ Yagi⁵⁾ and Maruyama *et al.*,⁶⁾ respectively. For proteolytic digestion, the sample was suspended in Tris buffer (0.1 M Tris-HCl-5 mM CaCl_2 , pH 8.0) at a concentration of approximately 0.5% and heated at 100°C for 5 min. Then trypsin or Pronase P was added to give an enzyme to substrate ratio of about 1:100 and digestion was allowed to proceed at 37°C for 12 hr with stirring with a magnetic stirrer. A small amount of thymol was added as an antibiotic agent. Exhaustive proteolytic digestion was performed by the sequential addition of Pronase P, carboxypeptidase A, prolidase and aminopeptidase M at 12 hr intervals essentially as previously described.⁷⁾

The identification and determination of citrulline in the digest of whole muscle was carried out as follows. The supernatant solution of the exhaustive proteolytic digest was fractionated on a column of Bio-Gel P-2 (2.1 $\times$ 91 cm) which had been calibrated previously with a standard amino acid. The citrulline fraction was collected and submitted to descending paper chromatography using *n*-butanol-acetic acid-water (4:1:1.7, v/v) for the preliminary separation of citrulline. The eluted solution containing citrulline from the paper chromatogram was analyzed with an amino acid analyzer with a Hitachi custom 2619 resin column (0.4 $\times$ 15 cm). Elution was carried out with 0.25 N lithium citrate buffer (pH 2.71) as previously described.⁸⁾ The proteolytic digest of whole muscle exhibited a

* Present address: Department of Microbiology, School of Medicine, Chiba University, Inohana, Chiba, Chiba 280, Japan.

** Present address: Tokiwa Gakuen College, Mito, Ibaraki 310, Japan.

peak that eluted at about 105 min. The peak was located between alanine (90 min) and valine (115 min). When authentic citrulline was added to the proteolytic digest of the whole muscle and analyzed, only the peak eluting at 105 min was increased and the extent of augmentation was as expected. When the acid hydrolysate (6 N HCl, 110°C, 16 hr) of the whole muscle was analyzed, similar results were obtained. For further confirmation of citrulline, the sample was hydrolyzed with 0.2 N potassium hydroxide at 110°C for 24 hr.⁹⁾ When the hydrolysate was analyzed with an amino acid analyzer, only ornithine which is derived from citrulline was detected and no lysine was observed. When the tryptic digest of whole muscle was chromatographed on a column of Bio-Gel P-2 (2.1 × 91 cm), citrulline color development¹⁰⁾ was only observed in the peptide fractions which appeared in the vicinity of the void volume and no free citrulline was detected. These results indicate the presence of citrulline in the protein of skeletal muscle of rabbit. The citrullyl residues of the protein in the muscle must have been derived from arginyl residues by the peptidylarginine deiminase present in the muscle, since the specific enzyme has been identified in rabbit skeletal muscle.¹⁾ Then the membranes, myosin, actin and connectin were prepared from the skeletal muscle of rabbit and citrulline contents of the enzymatic digests of these materials were estimated with an amino acid analyzer as described above. The results are summarized in Table I. The highest level of citrulline in the muscle was observed in the membranes and only small amounts of this amino acid were detected in myosin and actin. No citrulline was detected in connection which is an alkali insoluble elastic protein.

Protein bound citrulline in the skeletal muscle was found mainly in the membranes. Citrulline has also been detected in horny cell membranes of mammalian epidermis.⁸⁾ The amount of citrulline in the membranes of skeletal muscle was 7.53 μmol per gram of protein. Values in this range are less than those of horny cell membranes of the epidermis of

TABLE I. DISTRIBUTION OF CITRULLINE IN RABBIT SKELETAL MUSCLE

	μmol g protein ^a
Whole muscle	1.37 $\pm$ 0.03
Nonmembrane fraction	0.83 $\pm$ 0.02
Membranes	7.53 $\pm$ 0.03
Actin	0.01 >
Myosin	0.01 >
Connectin	0

^a Estimated by the ninhydrin method according to McGrath.¹¹⁾

newborn rat (140 μmol per g of protein) and cow snout (190 μmol per g of protein). The mammalian epidermis is a continuously differentiating tissue and during the differentiation, membranes of the cells thicken about twice and become biochemically inert. The presence of citrulline residues in the membranes of the muscle must be the result of deimination of arginyl residues of membrane protein or membrane-bound enzymes. It is postulated that the deimination of arginyl residues in protein will reduce the positive surface charge and allow the protein to fuse the membranes.

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REFERENCES

- 1) K. Sugawara, Y. Oikawa and T. Ouchi, *J. Biochem.*, **91**, 1065 (1982).
- 2) M. Kates, "Techniques of Lipidology," ed. by T. S. Work and E. Work, North-Holland, Amsterdam, 1972, pp. 330 ~ 340.
- 3) R. L. Barchi, J. B. Weigle, D. M. Chalikian and L. E. Murphy, *Biochim. Biophys. Acta*, **550**, 59 (1979).
- 4) W. F. H. M. Mommaerts, *J. Biol. Chem.*, **188**, 559 (1951).
- 5) K. Yagi, "Seikagaku Jikken Koza," Vol. 15, ed. by S. Ebashi and K. Maruyama, Tokyo Kagaku Dojin Co. Ltd., Tokyo, 1975, p. 4.
- 6) K. Maruyama, R. Natori and Y. Nonomura, *Nature*, **262**, 58 (1976).
- 7) K. Sugawara, *Agric. Biol. Chem.*, **43**, 2543 (1979).
- 8) K. Sugawara, *Agric. Biol. Chem.*, **43**, 2215 (1979).

- 9) G. R. Stark and D. G. Smyth, *J. Biol. Chem.*, **238**, 188 (1968).
214 (1963).
 - 10) G. Guthöhrlein and J. Knappe, *Anal. Biochem.*, **26**, 11) R. McGrath, *Anal. Biochem.*, **49**, 95 (1972).
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