THE PRESENCE OF CITRULLINE IN A MYELIN PROTEIN FRACTION

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1. Introduction

The amino acid citrulline is not generally found as a component of protein. However, Steinert, Harding and Rogers [1, 2] have identified citrulline as a constituent of proteins from the inner root sheath of hair follicles, and of proteins from cells of the medulla of hair fibers and porcupine quills. Rogers [1, 2] has isolated the L-citrulline component and identified it by IR spectroscopy, paper chromatography and oxidation with L-amino acid oxidase. He has also shown that citrulline can be released from the protein by digestion with subtilisin, and has identified citrulline-containing peptides.

In our work, citrulline was first found in acid hydrolysates of a protein fraction isolated from normal human myelin. This was confirmed by a direct colorimetric determination of citrulline in the non-hydrolysed protein. Pronase digestion of this protein material showed that citrulline was released from the protein. The released citrulline was separated on a Sephadex G-15 column and identified colorimetrically.

2. Methods

Normal human myelin was prepared according to the method of Lowden et al. [3] and a protein fraction was extracted with thioethanol [4]. Briefly, ten grams of myelin were stirred at 4° in 100 ml 0.014 M thioethanol overnight. The suspension was centrifuged at 10,000 rpm for 90 min in a Servall RC-2 centrifuge. The clear supernatant was saved and the pellet was washed twice with water. The water wash supernatants were pooled with the original extract and the whole was freeze-dried. The solid residue was then extracted in a soxhlet apparatus with chloroform–methanol (2:1) and dried over glycerol. This residue represents 5–10% of the weight of the myelin.

The thioethanol-soluble material was dissolved in phenol–acetic acid–water (3:1:1) in the proportion 1 mg protein/ml solvent and dialysed against 25% acetic acid to remove the phenol, followed by dialysis agianst formic-acetic buffer pH 1.9 (240 ml glacial acetic acid plus 60 ml 90% formic acid in 2 liters water). The resulting solution was centrifuged to remove any insoluble material and the protein concentration adjusted to 1.0 mg/ml.

Enzyme digestion of the thioethanol-soluble protein was carried out as follows. Pepsin was added to the dialysed protein solution in the proportion 1% w/w and incubated at 37° overnight. The mixture was freeze-dried to remove the formic-acetic buffer. The freeze-dried material was reconstituted in 0.1 M sodium phosphate buffer pH 7.0 to a concentration of 1.0 mg protein/ml. Pronase was added in a 1% w/w enzyme/protein ratio and the solution was incubated at 37°. The progress of pronase digestion was followed by taking a 1.0 ml sample of the digestion mixture at various times. The undigested protein was precipitated by adding 1.7 ml of 10% trichloroacetic acid (TCA) in the cold. The precipitate was sedimented by centrifuging and the supernatant was aspirated off. The precipitate was resolubilized in 1.0 ml of 2 N NaOH.

For the colorimetric assay of citrulline a combi-

nation of the methods of Snodgrass et al. [5], Strandjord et al. [6], and Ceriotti et al. [7] was used. Reagent A was 0.5% w/v diacetyl monoxime plus 15% w/v NaCl in water. Reagent B was 10 g antipyrine plus 2.5 g ferric ammonium sulfate dissolved in 500 ml H_2O to which 250 ml concentrated orthophosphoric acid and 250 ml concentrated sulfuric acid were added. To 1.0 ml of sample solution was added 1 ml of reagent A and 2 ml of reagent B. The samples were heated 15 min in a boiling water bath and then cooled. To samples containing TCA, 0.5 ml of ethylene glycol was added immediately after boiling [7]. The absorbance was read at 464 mμ in a spectrophotometer.

Protein concentration was determined by a modification of the ninhydrin method of Moore and Stein [8] using glutathione as a standard.

Gel permeation chromatography was carried out on a column of Sephadex G-15 measuring 1.5 X 50 cm. The gel was preconditioned according to the method of Goodson et al. [9] and then equilibrated with formic-acetic buffer pH 1.9. This buffer was also used to elute the column. Samples were applied in a 2 ml volume. Eighteen ml were collected followed by 2 ml fractions.

Samples of protein were prepared for amino acid analysis by hydrolysis in 5.7 N constant boiling HCl for 24 hr under nitrogen in a sealed vial. The hydrochloric acid was removed by evaporation on a Büchler rotary evaporator. The hydrolysed sample was applied to a Technicon long column. Since citrulline is known to be partially degraded to ornithine during acid hydrolysis [1], the values for citrulline and ornithine obtained were added together to give a more accurate value for total citrulline.

3. Results

Thioethanol-soluble protein purified by dialysis was found to contain citrulline both by amino acid analysis of the 5.7 N HCl hydrolysates and by direct colorimetric assay of the protein. Colorimetric analysis of the protein showed that there was 0.100 μmole citrulline per mg protein. Analysis of acid hydrolysates showed 0.094 μmole citrulline (0.065 μmole citrulline plus 0.029 μmole ornithine) per

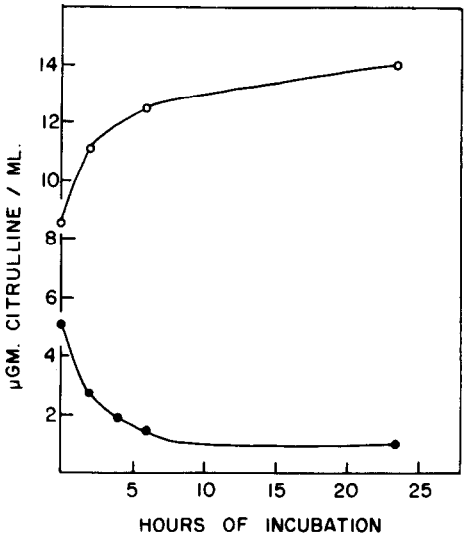

Fig. 1. Pronase digestion of thioethanol soluble protein. o——o TCA-soluble protein; •——• TCA-precipitated protein.

mg protein when run on the Technicon long column. The presence of citrulline in the acid hydrolysates was confirmed by chromatography in the Spinco system, which is different from that employed by Technicon. Whereas citrulline was eluted just after proline in the Technicon system, it appeared after glutamic acid in the Spinco system.

Since any citrulline loosely associated with the protein would have been removed on dialysis, it was necessary to look for further evidence that citrulline was bonded covalently in the molecule. Because the thioethanol-soluble protein was insoluble at pH 7.0 the protein was incubated first with pepsin at pH 1.9. The partially degraded protein was then soluble in 0.1 M sodium phosphate buffer pH 7.0. The progress of pronase digestion of the pepsin-treated protein was followed by sampling at various time intervals. Fig. 1. shows the release of citrulline into the TCA-soluble fraction and the loss of citrulline from the TCA-precipitated protein.

The proteolytic release of citrulline from the protein was confirmed by Sephadex G-15 gel filtration of the pepsin-pronase hydrolysate. Fig. 2A shows the elution pattern of the protein before digestion with pepsin and pronase. Fig. 2B shows the material eluted following enzyme treatment. It can be seen

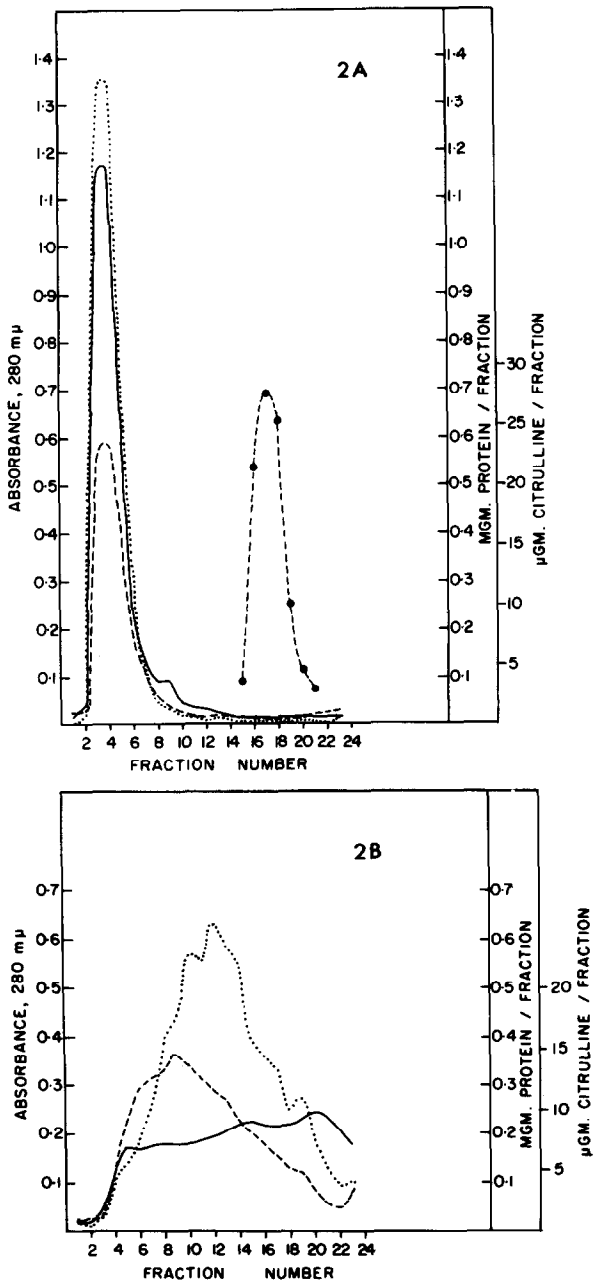

Fig. 2. Sephadex G-15 separation of protein. Column was 1.5 × 50 cm. Eighteen ml were collected, and then 2 ml fractions. The void volume was at fraction 5. A. Undigested protein B. Pepsin and pronase digested protein. Absorbance 280 mμ (——), ninhydrin (. . . . .), citrulline (- - - -), citrulline standard (● - - - ●).

that the protein had been completely degraded by the enzymes since none was recovered in the void volume. L-Citrulline, run as a standard on the same column, was eluted maximally in fraction 17 (fig. 2A).

4. Discussion

The results presented here show that citrulline is present in a protein fraction isolated from normal human myelin. Since the protein fraction had been subjected to soxhlet extraction, extensive dialysis, and gel filtration, it is unlikely that any free citrulline was present. In fact, passage of the protein through Sephadex G-15 showed that all the citrulline was recovered in the void volume while free citrulline was shown to be eluted much later in fraction 17.

The presence of citrulline in the void volume material was confirmed by two different column chromatographic techniques, the Technicon and Spinco systems. In the former, citrulline from acid hydrolysates of thioethanol-soluble protein was eluted just after proline while in the latter it was eluted after glutamic acid. Support for the presence of citrulline in the void volume material was obtained by the colorimetric assay.

By a combination of pepsin-pronase digestion citrulline was converted from high molecular weight material eluting in the void volume of the Sephadex G-15 column to low molecular weight material retarded on the same column. This suggests that citrulline was present in a covalent linkage in the protein.

From what is currently known about the incorporation of amino acids into protein it is unlikely that citrulline as such would be incorporated. It is more likely that modification of arginine takes place after incorporation. Modifications of amino acids after incorporation into protein such as the hydroxylation of proline to hydroxyproline in collagen are known to take place. Whether or not the presence of citrulline in a membrane protein has any functional significance remains to be determined.

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