BIKEN'S JOURNAL 2, 259-263 (1959)

Paper Electrophoresis and Detection on Paper of Glutamyl Polypeptide

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(Received for publication, December 15, 1959.)

SUMMARY

A new detection method for glutamyl polypeptide on paper is described. By paper electrophoresis and this detection method, glutamyl polypeptide was distinctly separated from protein and polysaccharide and easily detected on paper.

INTRODUCTION

Glutamyl polypeptide (GPP) was first found by Tomcsik and Szongott (1933) as a capsular material of *Bacillus anthracis*, and later it was also found in other species of the genus *Bacillus* (Ivánovics, 1937). This substance, which is a high molecular polypeptide linked by the γ-peptide bond of glutamic acid, has been noted by many workers because of its extraordinary structure and physiological behavior. So, a number of chemical, biochemical and microbiological investigations have been made on it (Bovarnick, 1942; Hanby and Rydon, 1946; Watson *et al.*, 1947; Waley, 1955; Thorne, 1956; Tomcsik, 1956; Zwartouw and Smith, 1956; Volcani and Margalith, 1957; Bruckner *et al.*, 1958; Chibnall *et al.*, 1958). In this laboratory, GPP has been studied with regard to the defence mechanisms of animals against *B. anthracis* (Torii, 1955, 1956, 1959a, 1959b; Amano *et al.*, 1958; Utsumi *et al.*, 1959a, 1959b).

In this report, a method of separation and detection of this substance by paper electrophoresis and precipitation by cupric sulfate is reported.

MATERIALS AND METHOD

1. *Materials*

Glutamyl polypeptides used were samples produced by *B. anthracis* "Vollum", *B. megaterium* "A5" and *B. subtilis*. *B. anthracis* and *B. megaterium* were kindly furnished by Prof. J. Tomcsik of University of Basel. They were grown and GPP was isolated as described in the previous paper (Torii, 1955). GPP of *B. subtilis* was kindly provided by Dr. M. Bovarnick of New York State University.

2. *Paper electrophoresis*

Toyo filter paper No. 51 (2 x 40 cm) was used. M/15 sodium acetate or M/10 acetic

acid-sodium acetate solution (pH 5.8) were used as the solvents. Samples dissolved in the buffer were pipetted onto filter paper previouly moistened with the same buffer. Electrophoresis was carried out for 2 or 3 hours at 500 volts.

RESULTS

Paper electrophoresis of GPP had been formerly carried out by Strange and Harkness (1953) and Nordberg and Thorsell (1955). As GPP is a polyanionic compound, the electrophoretic technique is a convenient method for separation of GPP from other neutral or basic materials. Therefore, this technique was also used in the present experiments.

For detection of GPP, the above authors used dyes. Rydon and Smith (1952) reported another method for detection of GPP on paper, involving exposure of the paper strip to chlorine gas and spraying it with potassium iodide-starch solu-

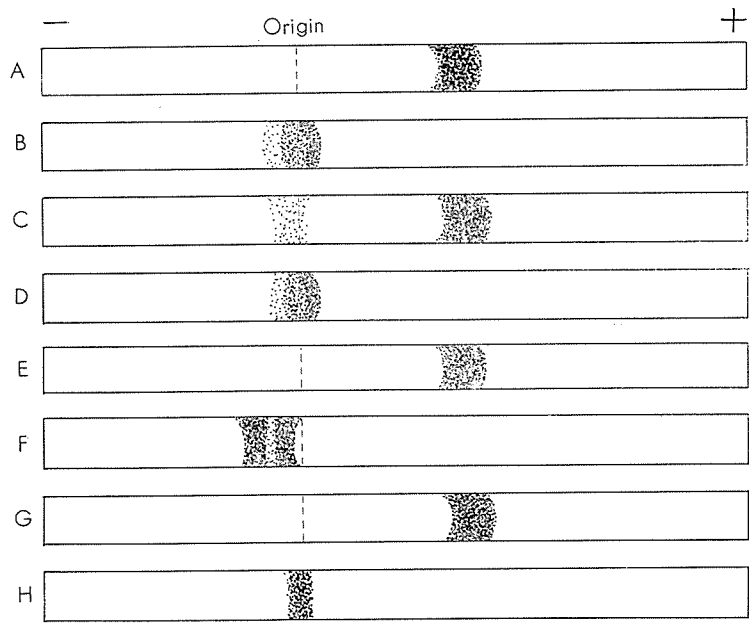

Fig. 1. Paper electrophoretic diagrams of GPP, and GPP mixed with protein or with polysaccharide.

A: GPP of *B. anthracis*, *B. megaterium* or *B. subtilis* 200μg ($CuSO_4$–$K_4Fe(CN)_6$ method)
B: Egg albumin 200μg (bromphenol blue staining)
C: GPP of *B. megaterium* 200μg+egg albumin 200μg ($CuSO_4$–$K_4Fe(CN)_6$ method)
D: GPP of *B. megaterium* 200μ+egg albumin 200μg (bromphenol blue staining)
E: GPP of *B. subtilis*+polysaccharide of *B. anthracis* ($CuSO_4$–K_4Fe $(CN)_6$ method)
F: GPP of *B. subtilis*+polysaccharide of *B. anthracis* (in an iodine atmosphere for detection of polysaccharide (Greenway *et al.*, 1953))
G: GPP of *B. subtilis*+soluble starch ($CuSO_4$–$K_4Fe(CN)_6$ method)
H: GPP of *B. subtilis*+soluble starch (in an iodine atmosphere)
A-D: M/10 acetic acid-sodium acetate buffer, pH 5.8
E-H: M/15 Sodium acetate solution

tion. But these procedures for its detection are troublesome and the colors which develop are not so stable. The method for the detection of GPP described below gives very stable color and the procedure is simpler.

1. *Color development*

After electrophoresis, the paper strips were dried and dipped in a solution of 2 per cent cupric sulfate for 3 minutes. Then the strips were thoroughly washed with water to remove excess cupric sulfate and dipped into 5 per cent potassium ferrocyanide. A brownish-red color appeared in the area containing the GPP. Three minutes later, the papers were washed and dried. The color was stable over several months. Although the minimum detectable amount of GPP was 10 μg, a good result was obtained with from 50 to 100 μg. When a large amount of the sample was applied, a light blue color was observed after washing the paper with water, even without dipping it into potassium ferrocyanide. The results of electrophoresis of GPP, and of GPP mixed with protein or with polysaccharide are summarized in Fig. 1.

2. *Confirmation of the presence of GPP in the colored area*

To ascertain the existence of GPP, the corresponding part of another paper was cut out and eluted with physiological saline. When this eluate was added to an anti GPP serum, obtained from a rabbit immunized with encapsulated *B. anthracis* (Utsumi *et al.*, 1959b), a serological specific precipitation was observed. Further, the eluate was hydrolysed with 6 N hydrochloric acid for 6 hours at 100° C, and the hydrolysate was tested by paper chromatography. Glutamic acid was shown to be the sole substance present.

3. *Chemical background of the reaction*

The mechanism of the reaction was first supposed as follows: the copper salt of GPP reacts with potassium ferrocyanide resulting in the formation of insoluble copper ferrocyanide and soluble potassium polyglutamate. The latter is released from the paper. However a test tube experiment showed an unexpected result. The copper salt derived from 2 mg of GPP of *B. megaterium* was thoroughly washed with water by centrifugation and treated with 2 ml of 5 per cent potassium ferrocyanide solution. The resulting brownish-red precipitate was washed with water by centrifugation and the material was hydrolysed with 6 N hydrochloric acid for 6 hours at 100°C. After removal of excess of hydrogen chloride, the residue was dissolved in water, neutralized with sodium hydroxide and treated with hydrogen sulfide to remove the copper. The copper free solution was concentrated and tested by paper chromatography. Glutamic acid was found. The brownish-red precipitate obtained from the copper polyglutamate and potassium ferrocyanide was somewhat soluble in water, while the same colored cupric ferrocyanide was insoluble. So, this brownish-red precipitate seemed to be not simple cupric ferrocyanide but a complex of copper polyglutamate and ferrocyanide ion.

DISCUSSION

Although the detection method described above showed as high a sensitivity

as that of the method of Rydon and Smith, there are some problems to be taken into account. Thus, it is unlikely that this color development is specific for GPP. Substances which produce insoluble precipitates with cupric ion will develop the same color. Further it must be remembered that certain buffer solutions, containing an anion such as phosphate which is precipitable with cupric ion, are unsuitable for electrophoresis.

By the method described here, GPP derived from three species of genus *Bacillus* showed the same electrophoretic behavior and were distinctly separated from protein and polysaccharide.

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