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PURIFICATION OF MONOTRICHOUS FLAGELLA OF *VIBRIO PARAHAEMOLYTICUS*

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SUMMARY A novel method to purify monotrichous flagella is described. For this, preparative zone electrophresis is used with a powdered co-polymer of polyvinyl chloride and polyvinyl acetate (Pevikon C-870) as supporting medium. The monotrichous flagella of *Vibrio parahaemolyticus* were purified by this method and an electron micrograph of the purified flagella was presented.

INTRODUCTION

Methods of purification of flagella have been developed by several workers. Weibull (1948) reported purification of the flagella of *Proteus vulgaris* by differential high-speed centrifugation after their mechanical remove from the organism. This method was used by Kobayashi et al. (1969) to prepare purified flagella of *Proteus vulgaris*, *Serratia marcescens* and *Bacillus subtilis*. This technique was used to purify flagella of *Salmonella typhimurium* and these flagella have been used in extensive studies on the structural components (Kerridge et al., 1962), antigenic substances (McDonough, 1965) and the reconstitution of flagella in vitro (Asakura et al., 1968). Martinez (1963) reported purification of the flagella of *Spirilum serpens*, *Proteus vulgaris* and *Bacillus subtilis* by ion exchange chromatography.

These methods were applied to obtain pure preparation of flagella from monotrichate bacteria such as *Vibrio metchnikovii* (Glauert et al., 1963) and *Vibrio fetus* (Keeler et al., 1966). However, preparations are frequently contaminated with spherical bodies.

In studies on the antigenicity of the flagella of *Vibrio parahaemolyticus*, a pathogenic rod with a single flagellum (Fujino et al., 1953; Fujino et al., 1965), our preparation were also contaminated with spherical bodies, which interfered with analysis of antigen.

This paper reports a new method for purification of flagella by preparative zone electrophoresis using a powdered co-polymer of polyvinyl chloride and polyvinyl acetate (Pevikon C-870) as supporting medium. Pure flagellar preparations free from spherical bodies were obtained in this way.

MATERIAL AND METHODS

1. *Strain and growth conditions*

The strain used was *Vibrio parahaemolyticus*

RIMD-100 (O1: Kl). Organisms were grown on nutrient agar containing 3% sodium chloride at 37C for 18 hr. They were harvested by washing them off with 3% sodium chloride solution.

2. *Preparation of partially purified flagella by differential high-speed centrifugation*

The cells were suspended in 3% sodium chloride solution and centrifuged at 16,000×*g* for 30 min. About 100 g of precipitated cells were resuspended in 400 ml of 3% sodium chloride solution. The flagella were removed from the cells using a Marusan Waring Blendor (8,000 rev/min for 5 min). The deflagellated bacteria were removed from the mixture by centrifugation at 16,000×*g* for 30 min. The flagella were precipitated by centrifugation at 70,000×*g* for 60 min. They were resuspended in 0.02 M borate buffer (pH 8.4) containing 3% sodium chloride and centrifuged at 28,000×*g* for 5 min. The preciptate was discarded and the flagella were precipitated by centrifugation at 70,000×*g* for 60 min. This washing procedure was repeated twice. The flagella were finally suspended in 0.02 M borate buffer (pH 8.4) containing 3% sodium chloride and designated as partially purified flagella (PPF).

3. *Purification procedure of PPF with DEAE-cellulose*

Ten grams of DEAE-cellulose (Brown, USA, capacity 0.85 mEq/g) was activated as described by Peterson and Sober (1962) and supended in 500 ml of 0.02 M borate buffer (pH 8.4). PPF (containing about 200 mg of protein) was added and the mixture was stirred for about 5 min and then filtered through a glass filter with suction. Flagella adsorbed on the DEAE-cellulose were retained on the filter. The filter was washed once with 600 ml of the above buffer and twice with 500 ml of 0.02 M borate buffer (pH 8.4) containing 0.2 M sodium chloride and the filtrates were discarded. Then the flagella adsorbed to DEAE-cellulose were eluted by washing with 0.02 M borate buffer (pH 8.4) containing 0.4 M sodium chloride.

4. *Zone electrophoresis*

Zone electrophoresis was carried out as described previously (Miwatani et al., 1969). A powdered co-polymer of polyvinyl chloride and polyvinyl acetate (Pevikon C-870) described by Mueller-Eberhard (1960) was used in place of potato starch as the supporting medium. Pevikon C-870 was washed 5 times with cold water and twice with borate buffer (pH 8.4, μ=0.05). A Pevikon block (40×7×1.5 cm) was prepared and electrophoretic separation was carried out at 4C with borate buffer (pH 8.4, μ=0.05). A current of 2 mA per square cm was applied for 14 hr and then the block was cut into sections of 1 cm width and each was eluted with 10 ml of the above borate buffer.

5. *Determination of protein*

The method of Lowry et al. (1951) was used for determination of protein.

6. *Determination of polysaccharide*

Polysaccharides were determined with Dreywood's Anthrone reagent as described by Morris (1948).

7. *Electron microscopy*

(a) *Shadowing.* Suspensions of samples in distilled water were placed on copper grids coated with Collodion. They were shadowed slightly with platinum-palladium.

(b) *Negative staining.* Sample suspensions in distilled water were mixed with an equal volume of 2% potassium phosphotungstate and placed on copper grids coated with Collodion films stabilized with a carbon layer.

The specimens were examined with a Hitachi electron microscope, Model 11 B.

RESULTS AND DISCUSSION

1. *Purification of flagella*

The electron micrograph in Fig. 1 shows bacteria with a monotrichous flagellum. Each flagellum has a sheath-like structure, as shown in Fig. 2. When bacteria grown on nutrient agar containing 3% sodium chloride at 37 C for 18 hr were harvested by washing them off with 3% sodium chloride solution, most of the sheath-like structure was removed. The flagellum without the sheath-like structure is shown in Fig. 3.

Flagella were removed from the cells by mechanical treatment as described in Material and Methods. Fig. 4 shows deflagellated cells, indicating that the mechanical treatment removed the flagella successfully.

Flagellar preparations were purified by differential high-speed centrifugation, as described in Materials and Methods, and the

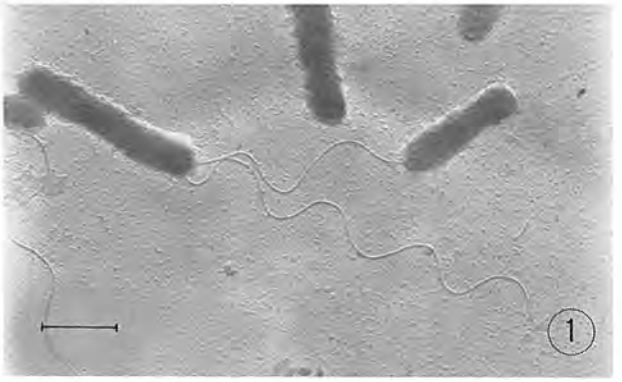

FIGURE 1. *Electron micrograph of Vibrio parahaemolyticus shadowing. Each cell has a monotrichous flagellum. The scale mark represents 1 μ.*

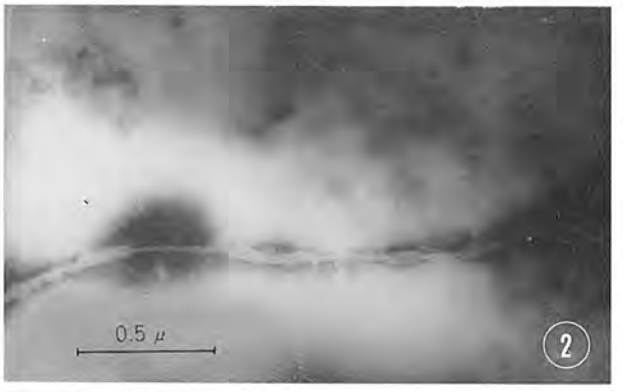

FIGURE 2. *Electron micrograph of the flagellum of Vibrio parahaemolyticus with negative staining. The flagellum has a sheath-like structure. The scale mark representes 0.5 μ.*

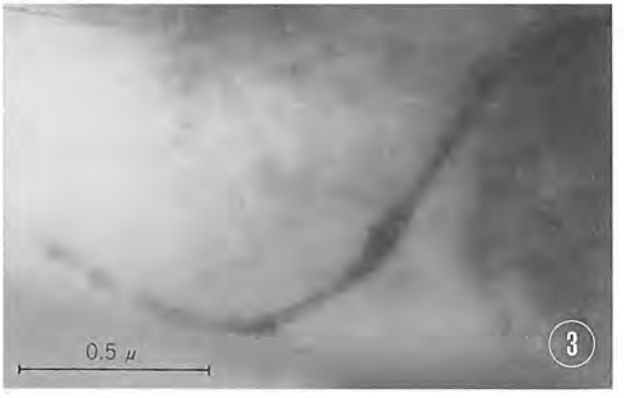

FIGURE 3. *Electron micrograph of flagellum without a sheath-like structure. Negative staining. The scale represents 0.5 μ.*

resulting partially pruified flagella (PPF) were photographed by electron microscopy. As shown in Fig. 5 this preparation was contaminated with spherical bodies. Keeler et al. (1966) also reported contamination of flagellar preparations from *Vibrio fetus* and Glauert et al. (1963) reported contamination of those of *Vibrio metchnikovii*. As stated by Keeler et al. (1966), the origin of these spherical bodies is unknown.

PPF was further purified using DEAE-cellulose, as described in Materials and Methods. Fig. 6 shows a typical electron micrograph of a flagellar preparation purified

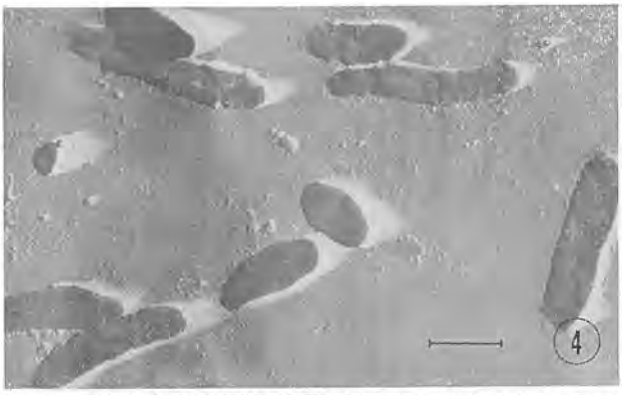

FIGURE 4. *Electron micrograph of deflagellated cells with shadowing. The scale represents 1 μ.*

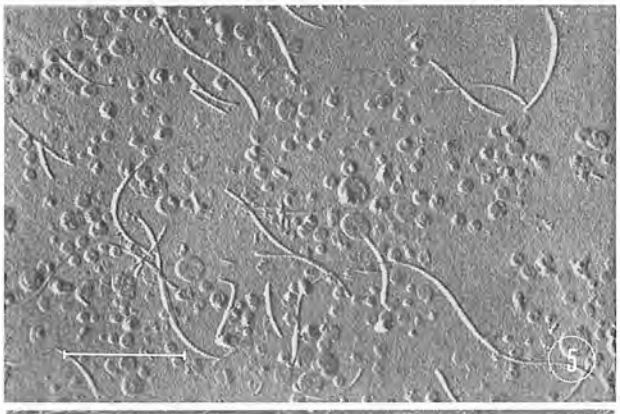

FIGURE 5. *Electron micrograph of PPF with shadowing. The flagella have no sheath. A large mass of contaminating spherical bodies is seen. The scale represents 1 μ.*

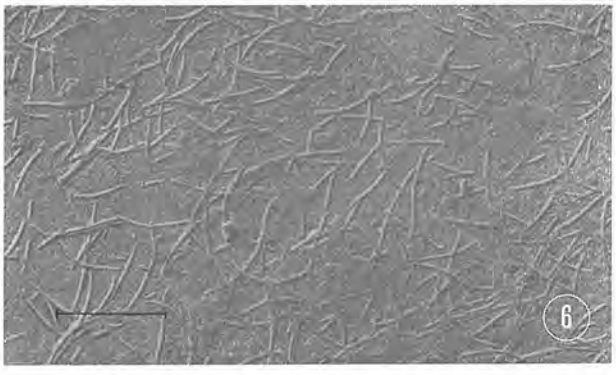

FIGURE 6. *Electron micrograph of DEAE-cellulose treated PPF with shadowing. Few spherical bodies are seen. The scale represents 1 μ.*

using DEAE-cellulose. Most, but not all, the contaminating spherical bodies had disappeared. Most of the spherical bodies were eluted from DEAE-cellulose with borate buffer (pH 8.4) containing 0.2 M sodium chloride. Fig. 7 shows an electron micrograph of the spherical bodies eluted from the DEAE-cellulose. Other methods of elution to separate the spherical bodies from the flagella were tested but were unsuccessful.

2. *Purification of PPF by zone electrophoresis*

PPF (equivalent to 30 mg of protein) was

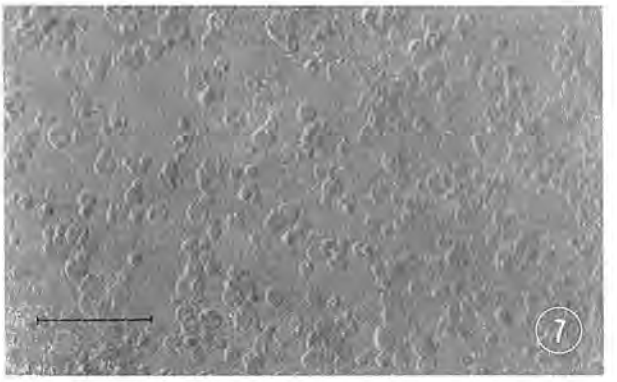

FIGURE 7. *Electron micrograph of spherical bodies with shadowing. DEAE-cellulose with adsorbed PPF was washed with borate buffer (pH 8.4) containing 0.2 M sodium chloride and the effluent was photographed. This fraction contains only spherical bodies. The scale represents 1 μ.*

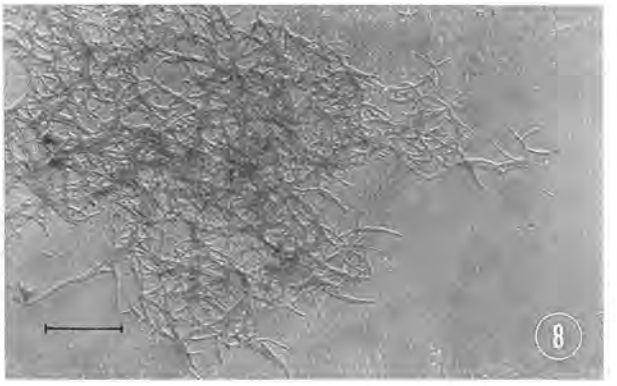

FIGURE 8. *Electron micrograph of purified flagella with shadowing. The scale represents 1 μ.*

subjected to preparative zone electrophoresis with a powdered co-polymer of polyvinyl chloride and polyvinyl acetate (Pevikon C-870) as the supporting medium, as described in Materials and Methods. Fig. 9 shows a typical profile obtained on zone electrophoresis. Four protein peaks were observed, three of which were contaminated with polysaccharide (Peak I, II and III). Material in peak IV was pooled and condensed by centrifugation and examined by electron microscopy. It was found that this was the flagellar fraction and that it was not contaminated with spherical bodies (Fig. 8). Electron microscopy showed that the other peaks contained spherical bodies but not flagella.

To study the structure and antigenicity of flagella, a pure flagellar preparation is required. Peritrichous flagella have been purified by several workers (Weibull, 1948; Kobayashi et al., 1959; Kerridge et al., 1962; McDonough, 1965), but there are no reports of successful purfication of the monotrichous flagella of vibrios. Using the new method described in this paper, it is now possible to prepare pure flagella from *Vibrio parahaemolyticus*. Studies on the antigenicity of the flagella of vibrios prepared by this method are now in progress and will be published elsewhere.

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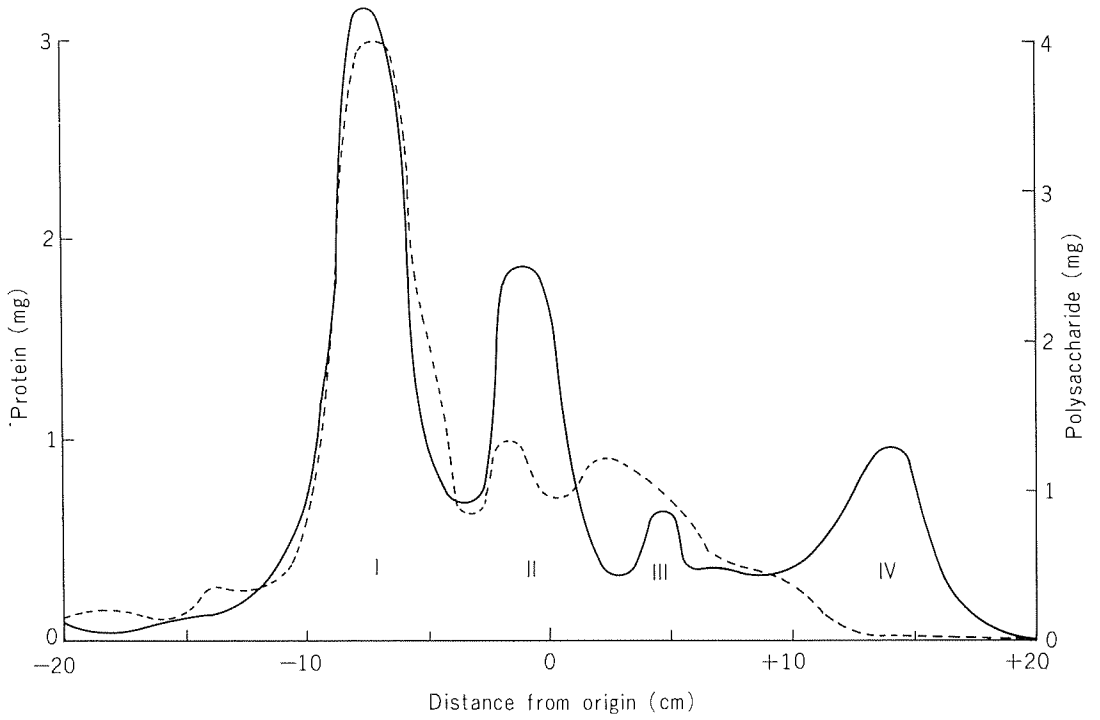

FIGURE 9. *Zone electrophoresis with Pevikon C-870 as supporting medium. PPF (equivalent to 30 mg of protein) was subjected to zone electrophoresis with Pevikon C-870 as supporting medium, as described in the text.*
—— *protein*; ..... *polysaccharide*

REFERENCES

Asakura, S., G. Eguchi and T. Iino. 1968. Unidirectional growth of Salmonella flagella in vitro. J. Mol. Biol. 35 : 227–236.

Fujino, T., Y. Okuno, D. Nakada, A. Aoyama, K. Fukai, T. Mukai and T. Ueho. 1953. On the bacteriological examination of Shirasu food poisoning. Med. J. Osaka Univ. 4 : 299–304.

Fujino, T., T. Miwatani, J. Yasuda, M. Kondo, Y. Takeda, Y. Akita, K. Kotera, M. Okada, H. Nishimune, Y. Shimizu, T. Tamura and Y. Tamura. 1965. Taxonomic studies on the bacterial strains isolated from cases of " Shirasu " food poisoning (*Pasteurella parahaemolytica*) and related microorganisms. Biken J. 8: 63–71.

Glauert, A. M., D. Kerridge and R. W. Horne. 1963. The fine structure and mode of attachment of the sheathed flagellum of *Vibrio metchnikovii*. J. Cell Biol. 18: 327–336.

Keeler, R. F., A. E. Ritchie, J. H. Bryner and J. Elmore. 1966. The preparation of cell walls and the preparation of flagella of *Vibrio fetus*. J. Gen. Microbiol. 43 : 439–454.

Kerridge, D., R. W. Horne and A. M. Glauert. 1962. Structural components of flagella from *Salmonella typhimurium*. J. Mol. Biol. 4: 227–238.

Kobayashi, T., J. N. Rinker and H. Koffler. 1969. Purification and chemical properties of flagellin. Arch. Biochem. Biophys. 84 : 342–362.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 : 265–276.

Martinez, R. J. 1963. A method for the purification of bacterial flagella by ion exchange chromatography. J. Gen. Microbiol. 33 : 115–120.

McDonough, M. W. 1965. Amino acid compo-

sition of antigenically distinct Salmonella flagellar proteins. J. Mol. Biol. 12 : 342–355.
Miwatani, T., S. Shinoda, H. Nishimune, M. Okada, Y. Takeda and T. Fujino. 1969. A common antigenic substance of *Vibrio parahaemolyticus*. I. Isolation and purification. Biken J. 12 : 97–106.
Morris, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science 107 : 254–255.
Mueller-Eberhard, H. J. 1960. A new supporting medium for preparative electrophresis. Scand. J. Clin. Lab. Invest. 12 : 33–37.
Peterson, E. A. and H. A. Sobar. 1962. Column chromatography of proteins : substituted cellulose. p. 3–27. In S. P. Colowick and N. O. Kaplan [ed.] Methods in Enzymology. Academic Press. New York and London.
Weibull, C. 1948. Some chemical and physicochemical properties of the flagella of *Proteus vulgaris*. Biochim. Biophys. Acta 2 : 351–361.