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ANTIGENS OF *VIBRIO PARAHAEMOLYTICUS*. II. EXISTENCE OF TWO DIFFERENT SUBUNITS IN THE FLAGELLA OF *VIBRIO PARAHAEMOLYTICUS* AND THEIR CHARACTERIZATION

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SUMMARY 1. The physical and chemical properties of the monotrichous flagella of *Vibrio parahaemolyticus* were studied.

2. The flagella of *Vibrio parahaemolyticus* were solubilized by treatment with either 6 M urea, 0.1% acetic acid, 0.2% sodium dodecyl sulfate, 0.2% sodium dodecyl benzyl sulfate or heat (65 C, 3 min).

3. The flagella were separated in two distinct subunits (U-1 and U-II) by hydroxylapatite column chromatography.

4. U-I and U-II were demonstrated to differ antigenically.

5. U-I and U-II both a molecular weight of approximately 40,000.

6. Most physico-chemical properties of U-I and U-II were similar, but their amino acid compositions were different.

INTRODUCTION

Physical and chemical properties of the subunits of flagella (flagellins) of various bacteria have been throughly studied by several workers. Studies on the flagellins of *Salmonella* (Weibull, 1950; Asakura et al., 1964), *Spirillum serpens* (Martinez et al., 1967) and *Proteus vulgaris* (Weibull, 1950; Chang et al., 1969) showed that the flagellins of these bacteria appear to be single proteins with a molecular weight of about 40,000.

These bacteria possess a peritrichous flagella, and it is interesting to compare the physicochemical properties of the subunits of these flagella with those of monotrichous flagella. Recently, we reported a method for purification of monotrichous flagella of *Vibrio parahaemolyticus* (Miwatani et al., 1970). Using flagella of *Vibrio parahaemolyticus* purified by this method we characterized the flagellins of *Vibrio parahaemolyticus*. It was found that the flagella of *Vibrio parahaemolyticus* contain two distinct submits, both having a molecular weight of about 40,000.

MATERIALS AND METHODS

1. *Strains*

Vibrio parahaemolyticus RIMD-100 (O1 : K1) from the stock culture in the Type Culture Collection of the Research Institute for Microbial Diseases, Osaka University was used throughout.

2. *Preparation of flagella*

Growth conditions and purification of flagella by preparative zone electrophoresis using a powdered co-polymer of polyvinyl chloride and polyvinyl acetate (Pevikon C–870) as supporting medium were described previously (Miwatani, et al., 1970).

3. *Sephadex G–100 column chromatography*

Sephadex G–100, super fine (Pharmacia, Sweden) was suspended in an appropriate amount of 0.01 M phosphate buffer (pH 7.0) and the swollen gel was allowed to settle by gravity to make columns (2 × 125 cm). Columns were eluted with 0.01 M phosphate buffer (pH 7.0) and 5 ml fractions were collected. Chromatography was carred out in a cold room at 4 C.

4. *Hydroxylapatite column chromatography*

The purified preparaction of flagella (about 10 mg) was treated with 6 M urea and applied to a hydroxylapatite column (1 × 20 cm) previously equilibrated with 0.001 M phosphate buffer, pH 7.0 Hydroxylapatite was prepared by the method by Levin (1962). The column was eluted with 100 ml of the above buffer, and then with a linear gradient of 0.01 M to 0.2 M phosphate buffer, pH 7.0. A total volume of 160 ml of buffer was used for elution, and 6 ml fractions were collected. All procedures were carried out in a cold room at 4 C.

5. *Determination of protein*

Protein was usually determined by the method of Lowry et al. (1951). The protein content of fractions eluted from some columns was determined from the absorption at 280 mμ.

6. *Preparation of antiserum of flagellin*

Freund's incomplete adjuvant was used for preparation of immunizing antigens. About 500 μg of protein (flagellin or flagella) suspended in 5 ml of 0.01 M phosphate buffer (pH 7.0) were used as antigens and emulsified with an equal volume of a mixture of Arlacel A (Atlas Powder Co.) and Drackeol No. 6 (Pennsylvania Industries). Arlacel A and Drackeol No. 6 were mixed in the ratio of 1.5: 8.5, v/v. The immunizing antigens were inoculated intramuscularly into young male rabbits, weighing 2.5 to 3 kg. A booster injection was made after 4 weeks. Antisera were obtained one week after the last injection.

7. *Gel diffusion test*

The gel diffusion test was carried out as described previously (Miwatani et al. 1969).

8. *Immunoelectrophoresis*

Immunoelectrophoresis was carried out as described previously (Miwatani et al., 1969).

9. *Determination of nitrogen content*

For determination of the nitrogen content, the sample was digested with sulfuric acid and hydrogen peroxide and assayed with ninhydrin as described by Schiffman et al. (1964).

10. *Determination of sugar content*

The sugar content was determined with Dreywood's Anthrone reagent as described by Morris (1948).

11. *Determination of phosphorous content*

The phosphorous content was determined by the method of Allen (1940).

12. *Analytical centrifugation*

A solution of flagellin (1 mg/ml) in 0.01 M phosphate buffer (pH 7.0) was centrifuged in a Hitachi Analytical Centrifuge, model UCA-1A. Runs were made at 55,430 rev/min and pictures were taken at ten minutes intervals after reaching this speed.

13. *Amino acid analysis*

The amino acid compositions of flagellins were determined in a Yanagimoto, model LC–50, amino acid analyzer. Five mg of sample were hydrolyzed by heating in 2 ml of 6 N HCl at 105 C for 24 hr in a sealed tube. Then the mixture was dried by evaporation and dissolved in 2 ml of 0.2 M citrate buffer (pH 2.2). A 0.5 ml aliquot was examined in the analyzer.

RESULTS

1. *Solubilization of purified flagella*

In attempts to solubilize the purified flagella, several treatments were applied to suspensions of flagella in 0.01 M phosphate buffer (pH 7.0) containing 3% sodium chloride. These were; (A) treatment by urea: a flagellar suspension was kept in 6 M urea for 60 min at 37 C, (B) treatment by acetic acid: a flagellar suspension was kept in 0.1 M acetic acid for 60 min at 37 C, (C) treatments by various detergents: flagellar suspensions were kept in 0.2% of either sodium dodecyl sulfate, sodium dodecyl benzyl sulfate or Triton X-100, for 60 min at 37 C, and (D) heat treatment: a flagellar suspension was kept at 65 C for 1, 3 or 5 min. The final concentration of flagella was adjusted to 1 mg per 1 ml of reaction mixture. After these treatments, the flagellar suspensions were centrifuged at 100,000 × *g* for 60 min and the concentration of protein in the supernatant was determined. As shown in Table 1, almost all the protein was recovered after treatment with urea, acetic acid, sodium dodecyl sulfate or sodium dodecyl benzyl sulfate, indicating that the flagella were solubilized successfully by these treatments. Heat treatment at 65 C for 3 min or more also solubilized the flagella.

TABLE 1. *Solubilization of purified flagella*

Treatment	Protein concentration in supernatant (mg/ml)
6 M urea	0.98
0.1 M acetic acid	0.92
0.2 % SDS	1.02
0.2 % SDBS	0.98
0.2 % Triton X-100	0.79
65 C 1 min	0.83
„ 3	0.92
„ 5	0.99

Suspensions of purified flagella of Vibrio parahaemolyticus (1 mg/ml) in 0.01 M phospate buffer (pH 7.0) containing 3 % sodium chloride were treated as described in the text. After centrifugation at 100,000 × g for 60 min, the protein concentration in the supernatant was determined.

The antigenicities of the flagellins solubilized by these treatments were compared and the results are shown in Fig. 1. Antiserum to the flagellin obtained by treatment with 6 M urea was placed in the center well and the flagellins obtained with urea, acetic acid, sodium dodecyl sulfate and heat (65 C, 3 min) were used as antigens. It was found that all antigens were identical, indicating that the antigenicity was not changed by these treatments.

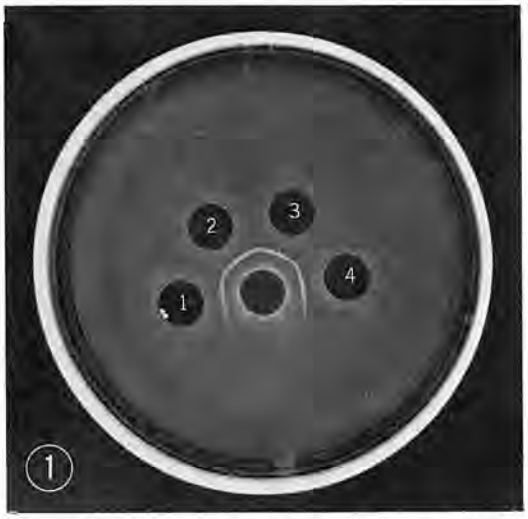

FIGURE 1. *Antigenicities of flagellins of Vibrio parahaemolyticus solubilized by various treatments.*

The gel diffusion test was carried out as described in the text. Antiserum to the flagellin obtained by treatment with 6 M urea was placed in the center well. Flagellins obtained with sodium dodecyl sulfate, urea, acetic acid and heat (65 C, 3 min) were placed in wells 1, 2, 3 and 4 respectively.

2. *Isolation of two different subunits*

Immunoelectrophoresis of urea treated flagella (Fig. 2) suggests that they consist of two antigenically different substances, so attempts were made to isolate different subunits. As shown in Fig. 3, on hydroxylapatite column chromatography, two different subunits were obtained from purified flagella, one being eluted with about 0.03 M phosphate buffer and the other with about 0.1 M phosphate buffer. These subunits were tentatively designated as U-I and U-II, respectively.

FIGURE 2. *Immunoelectrophoresis of flagellin.*
Immunoelectrophoresis was carried out as described in the text. Flagellin obtained by treatment with 6 M urea was placed in the well. Antiserum to the flagellin obtained with 6 M urea treatment was placed in the through.

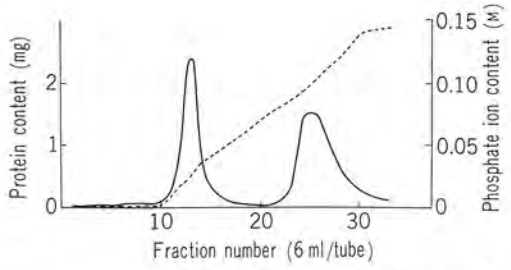

FIGURE 3. *Hydroxylapatite column chromatogram of the flagellin of Vibrio parahaemolyticus.*
Hydroxylapatite column chromatography was carried out as described in the text. Protein content was determined by the method of Lowry et al. (1951). Phosphate ion content was determined by the method of Allen (1940).

3. *Antigenicities of subunits, U-I and U-II*

To verify that these two subunits were not identical substances, their antigenicities were studied. The results of a gel diffusion test, seen in Fig. 4A, clearly show that the precipitation line between antiflagellin and U-I crossed the precipitation line between antiflagellin and U-II, indicating that U-I and U-II differ antigenically. This was confirmed by gel diffusion tests using anti-U-I and anti-U-II (Fig. 4B). No precipitation line was observed between anti-U-I and U-II, or between anti-U-II and U-I. These findings suggest that the flagella of *Vibrio parahaemolyticus* consist of two antigenically different subunits.

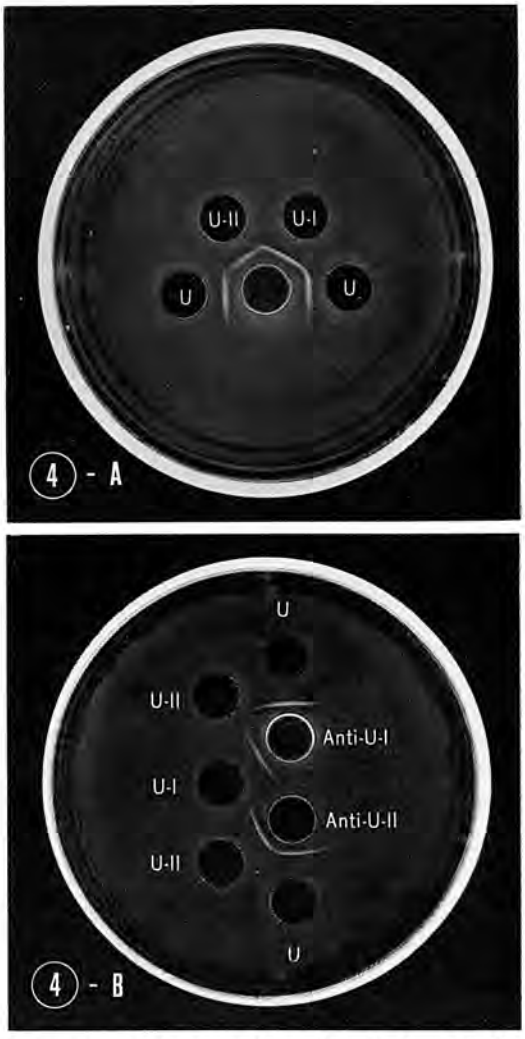

FIGURE 4. *Antigenicity of flagellins, U-I and U-II.*
The gel diffusion test was carried out as described in the text. Antisera to urea treated flagella, U-I and U-II were placed in the wells marked anti-U, anti-U-I and anti-U-II, respectively. Urea treated flagella, U-I and U-II were placed in the wells marked U, U-I and U-II, respectively.

4. *Physicochemical properties of U-I and U-II*

To determine the molecular weights of the subunits, they were subjected to gel filtration and results are shown in Fig. 5. Appropriate amounts of U-I, U-II and flagellin were applied separately to Sephadex G-100 columns

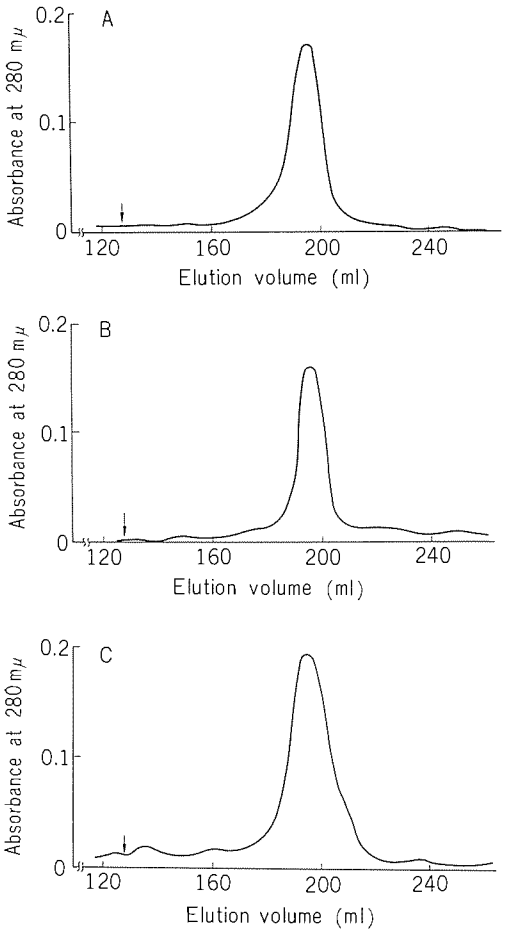

FIGURE 5. *Gel filtration of flagellins of Vibrio parahaemolyticus. Sephadex G-100 gel filtration of U-I (A), U-II (B) and urea treated flagella (C) was carried out as described in the text. The arrow indicates the void volume.*

and the columns were eluted as described above. Typical chromatograms of U-I (Fig. 5A) and U-II (Fig. 5B) showed that these two subunits have a similar molecular weight. Consequently, the chromatogram of flagellin which contained U-I and U-II also had a similar profile (Fig. 5C). From comparison of column chromatograms of bovine serum albumin and ovalbumin, the molecular weight of these flagellins was determined to be about 40,000.

Other physicochemical properties of U-I and U-II are summarized in Table 2. These results show no significant differences between U-I and U-II. However, the results of amino acid analysis seen in Table 3, show that the amino acid compositions of these flagellins are not identical. Significant differences were observed in the lysine, aspartic acid, glutamic acid, alanine, valine and methionine contents.

TABLE 2. *Physicochemical properties of flagellins, U-I and U-II, of Vibrio parahaemolyticus*

Property	U-I	U-II
Nitrogen content (%)	16.4	16.1
Sugar content (%)	0.04	0.02
Phosphorous content (%)	0.01	0.01
S value	2.6 S	2.7 S
A_{280}/A_{260}	1.61	1.59

Properties were determined as described in the text.

TABLE 3. *Amino acid compositions of flagellins, U-I and U-II*

Amino acid	U-I	U-II
Lysine	16.4	25.6
Histidine	3.2	1.8
Arginine	12.5	8.4
Aspartic acid	47.5	59.0
Threonine	28.6	24.4
Serine	26.6	30.7
Glutamic acid	36.9	56.0
Proline	1.2	0
Glycine	36.6	41.0
Alanine	57.3	38.8
Cystine	0	0
Valine	10.0	27.4
Methionine	10.7	2.6
Isoleucine	20.7	23.8
Leucine	26.2	25.2
Tyrosine	3.2	5.4
Phenylalanine	10.0	8.8
Amide nitrogen	37.8	39.0

Amino acid analysis of U-I and U-II were carried out as described in the text. Values are expressed in moles/mole protein.

DISCUSSION

There are several reports on the physical and chemical properties of peritrichous flagella of various bacteria (Weibull, 1950; Asakura et al., 1964; McDonough, 1965; Martinez et al., 1967; Chang et al., 1969). However, little is known about the physical and chemical properties of monotrichous flagella. This is because monotrichous flagella have not been purified. Several attempts were made to purify the monotrichous flagella of *Vibrio metschnikovii* (Glauert et al., 1963) and *Vibrio fetus* (Keeler et al., 1966) but the resulting flagellar preparations were contaminated with spherical bodies. Recently, we developed a method to purify monotrichous flagella uncontaminated with spherical bodies (Miwatani et al., 1970), which made it possible to study the physical and chemical properties of monotrichous flagella.

Various treatments previously reported for solubilization of flagella were applied to the flagella of *Vibrio parahaemolyticus*. Urea (6 M), acetic acid (0.1 M), sodium dodecyl sulfate (0.2%), sodium dodecyl benzyl sulfate (0.2%) and heat (65 C, 3 min) solubilized the flagella of *Vibrio parahaemolyticus* completely. Similar findings have been reported (Roberts and Doetsch, 1966). These treatments did not change the antigenicity of the flagellins.

It is interesting that two different subunits were found in the flagella of *Vibrio parahaemolyticus*. The various bacterial flagella so far reported all consist of a single subunit. In *Salmonella*, flagellar antigens are immunologically multiple. However, attempts to isolate different subunits were unsuccessful (Weibull, 1950; Asakura et al., 1964; McDonough, 1965). The two subunits of *Vibrio parahaemolyticus* were isolated by hydroxylapatite column chromatography. These two subunits were demonstrated to differ antigenically.

Most of the physicochemical properties of these two subunits were quite similar. Their molecular weights were identical being approximately 40,000. This value is the same as that reported previously for peritrichous flagella (Weibull, 1948; Kobayashi et al., 1959; Martinez et al., 1967; Chang et al., 1969). However, amino acid analysis showed that these two subunits were not identical.

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