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A COMMON ANTIGENIC SUBSTANCE OF *VIBRIO PARAHAEMOLYTICUS*

I. ISOLATION AND PURIFICATION

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SUMMARY An antigenic substance of *Vibrio parahaemolyticus* was isolated and designated as A-substance. This substance was common to strains of *Vibrio parahaemolyticus* but did not exist in other vibrios such as *Vibrio cholerae* and *Vibrio alginolyticus*. This substance was found to be different from O-, K-, and flagellar-antigens of *Vibrio parahaemolyticus*.

This substance was purified by DEAE-cellulose column chromatography, Sephadex G-200 gel filtration and starch-block electrophoresis. Purified A-substance was demonstrated to be a single component by analytical centrifugation, gel diffusion and immunoelectrophoresis. A-substance was found to be present in all 126 strains of *Vibrio parahaemolyticus* isolated from clinical sources.

INTRODUCTION

There have been reports from several laboratories on the antigenic structure of *Vibrio parahaemolyticus* and the existence of O-, K-, and flagellar-antigen in this organism have been described (Sakazaki, 1965; Omori et al., 1966; Sakazaki et al., 1968; Terada, 1968; Miwatani et al., 1969). During studies on antigens of *Vibrio parahaemolyticus*, we found a novel antigenic substance common to strains of this organism and differing from previously reported antigens such as O-, K-, and flagellar-antigen. This paper reports the isolation and purification of this antigenic substance common to strains of *Vibrio parahaemolyticus*.

1 Supported in part by grants from the Ministry of Education of Japan and the Ministry of Welfare of Japan.

MATERIALS AND METHODS

1. *Strains*

The bacterial strains used in this experiment were as follows: 1) *Vibrio parahaemolyticus* EB 101, the type culture of this species (Fujino et al., 1953), 12 pilot strains representing the serotypes O-1–O-10, O-N1 and O-N2 (Miwatani et al., 1969) and 126 strains isolated from clinical sources. 2) Other

vibrios; 5 strains of *Vibrio cholerae*, 5 strains representing Gardner-Venkatraman's serotypes II (NCTC 8042), III (NCTC 4711 and 30), V (NCTC 4715) and VI (NCTC 4716), 6 strains representing Heiberg's biological types and 5 strains of *Vibrio alginolyticus* (Sakazaki, 1968).

2. *Preparation of Fraction A containing an antigenic substance common to strains of Vibrio parahaemolyticus.*

Vibrio parahaemolyticus cells were inoculated onto nutrient agar containing 3% sodium chloride and cultured at 37 C for 15 hr. Cells were collected and suspended in 3% sodium chloride solution (100 g wet weight of cells per 400 ml of solution). This suspension was homogenized in a Marusan homoblendor (Sakuma Seisakusho) at 8,000 rpm for 5 min and centrifuged at $16,000 \times g$ for 30 min. This treatment was repeated. Flagella of cells were removed from the organisms by these treatments and were present in the supernatant (sup-1) obtained by centrifugation. The cells collected by centrifugation were suspended in 400 ml of 3% sodium chloride solution. The suspension was shaken with glass beads (6 mm in diameter) at 37 C for 16 hr (or sometimes for 1 hr) and cells were removed by centrifugation ($16,000 \times g$ for 30 min). The resulting supernatant fluid (sup-2) containing the antigenic substance was used for further treatment. Some of antigenic substance was present in sup-1, so this was centrifuged at $70,000 \times g$ for 60 min to remove flagella and mixed with sup-2. Solid ammonium sulfate was added to this supernatant fluid to 80% saturation, and the mixture was stood for 15 hr at 4 C and their centrifuged at $16,000 \times g$ for 30 min. The precipitate was dissolved in 0.01 M phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer at 4 C. It was designated as Fraction A. For vibrios other than *V. parahaemolyticus*, the concentrations of sodium chloride used for cultivation and for preparing the cell suspensions were 0.5% and 0.9%, respectively.

3. *Fractionation of Fraction A with ammonium sulfate*

Saturated ammonium sulfate solution (pH 7.0) was added to Fraction A of *V. parahaemolyticus* to give 30% saturation, and the mixture was stood for 3 hr at 4 C and then centrifuged at $16,000 \times g$ for 30 min. The precipitate was discarded, and additional saturated ammonium sulfate solution (pH 7.0) was added to the supernatant fluid to 45% saturation. The mixture was stood overnight at 4 C and then centrifuged at $16,000 \times g$ for 30 min. The resulting precipitate was suspended in a small volume of 0.01 M phosphate buffer (pH 7.0), and dialyzed overnight against the same buffer at 4 C.

4. *DEAE-cellulose column chromatography*

DEAE-cellulose (Brown Co., USA, capacity 0.85 mEq/g) was activated as described by Peterson and Sober (1962) and buffered with 0.01 M phosphate buffer (pH 7.0). Columns (2×20 cm) were prepared by allowing the buffered DEAE-cellulose to settle by gravity. Generally, 500 mg of protein were applied on each column and eluted stepwise with 0.01 M phosphate buffer (pH 7.0) containing different concentrations of sodium chloride at a flow rate of 30 ml per hour, collecting 10 ml fractions. Chromatography was carried out in a cold room at 4 C.

5. *Sephadex G-200 column chromatography*

Sephadex G-200 (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 (4×100 cm). Elution was done with 0.01 M phosphate buffer (pH 7.0) and 15 ml fractions were collected. Chromatography was carried out in a cold room at 4 C.

6. *Starch-block electrophoresis*

Starch-block electrophoresis was carried out essentially as described by Kunkel and Slater (1952). Potato starch used as the supporting medium was washed 5 times with cold distilled water and twice with borate buffer (pH 8.4, $\mu = 0.05$). A starch block ($40 \times 7 \times 1.5$ cm) was prepared and electrophoretic separation was carried out at 4 C with borate buffer (pH 8.4, $\mu = 0.05$). A current of 2 mA per cm^2 was applied for 16 hr and then the starch block was cut into sections of 1 cm width and each was eluted with 10 ml of the borate buffer described above.

7. *Preparation of antiserum of the common antigenic substance of V. parahaemolyticus*

Freund's incomplete adjuvant was used for preparation of immunizing antigens for the first inoculation. Fraction A (10 mg protein per ml) or DEAE-cellulose fractionated material (Fraction PPA, 1–2 mg protein per ml) was used for 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. These immunizing antigens were inoculated intramusclarly into young male rabbits weighing 2.5 to 3 kg. After 2 weeks, intravenous booster injections of either Fraction A or Fraction PPA were made at 4 days intervals, and successively 0.2, 0.4, 0.6, 0.8 and 1.0 ml of antigen solution were inoculated at each booster injection. One week after the last injection, antisera were obtained, inactivated by treatment at 56 C for 30 min and kept frozen until used.

8. *Gel diffusion test*

The plate method of Ouchterlony (1949) was used for the gel diffusion test. Difco Special Agar-Noble in 0.01 M phosphate buffer (pH 7.0) at a concentration of 0.7% was used and thimerosal (0.02%) was added as an antiseptic. Samples were put into the wells and the plates were placed in a humidified incubator (37 C). The precipitin reactions were observed and photographed after 2 to 5 days incubation.

9. *Immunoelectrophoresis*

A layer of agar gel (0.7% Difco Special Agar-Noble in borate buffer pH 8.4, $\mu=0.025$, containing 0.02% thimerosal) was prepared on a glass plate. A small hole was cut in the agar gel and the antigen solution was put into it. Electrophoresis was carried out for 2 hr (10 mA/7 cm). Then an appropriate antiserum was placed in the trough and the diffusion precipitin reaction was carried out in a humidified incubator at 37 C for 15 hr.

10. *Determination of protein*

Protein was determined by the method of Lowry et al. (1951) For chromatography, the protein content of each fraction was determined by measuring the absorption at 280 mμ.

11. *Analytical centrifugation*

The solution of purified A-substance (5 mg/ml) in 0.01 M phosphate buffer (pH 7.0) was centrifuged in a Hitachi Analytical Centrifuge, model UCA-1A at 55,430 rpm and the picture was taken 30 min after reaching maximal.

12. *Preparation of crude antigen to test the existence of A-substance in V. parahaemolyticus from clinical sources*

Bacteria were grown on a nutrient agar slant containing 3% sodium chloride at 37 C for 18 hr and then suspended in 2 ml of 3% sodium chloride solution. A little toluene were dropped into the solution, which was then kept at 37 C overnight. It was used as a crude antigen for the gel diffusion test.

RESULTS

1. *Serological specificity of Fraction A of Vibrio parahaemolyticus EB* 101

Agar gel diffusion precipitation was carried out to study the serological specificity of Fraction A of *V. parahaemolyticus* EB 101 and the results is shown in Fig. 1. The antiserum of Fraction A of *V. parahaemolyticus* EB 101 gave a clear common precipitation line with Fraction A from all the different strains of *V. parahaemolyticus* tested (EB 101, O-1—O-10) (Fig. 1–1, 1–2, 1–3), although the antiserum gave several different precipitation lines not only with homologous Fraction A (EB 101) but also with Fraction A of other strains of *V. parahaemolyticus* (O-1—O-10). On the other hand, this antiserum did not give any common precipitation line with Fraction A of 2 strains of *V. alginolyticus* (Fig. 1–3), 5 strains of vibrios representing Gardner-Venkatraman's serotypes II, III, V and VI (Fig. 1–4), 5 strains of *V. cholerae* including El Tor type, and 6 strains of vibrios representing Heiberg's biological types. This result indicates that there is an antigenic substance common to different strains of *V. parahaemolyticus* which is not present in other vibrios such as *V. cholerae* and *V. alginolyticus*. This common antigenic substance present in Fraction A of *V. parahaemolyticus*, and responsible for the common precipitation line on the agar gel, was designated as A-substance.

2. *Purification of A-substance*

Fraction A of *V. parahaemolyticus* O-1 was prepared as described in Materials and Methods, and used for further purification of A-substance. Using the gel diflusion test, it was found that A-substance could be fractionated by ammonium sulfate treatment and precipitated at 30–45% saturation of salt. This fraction

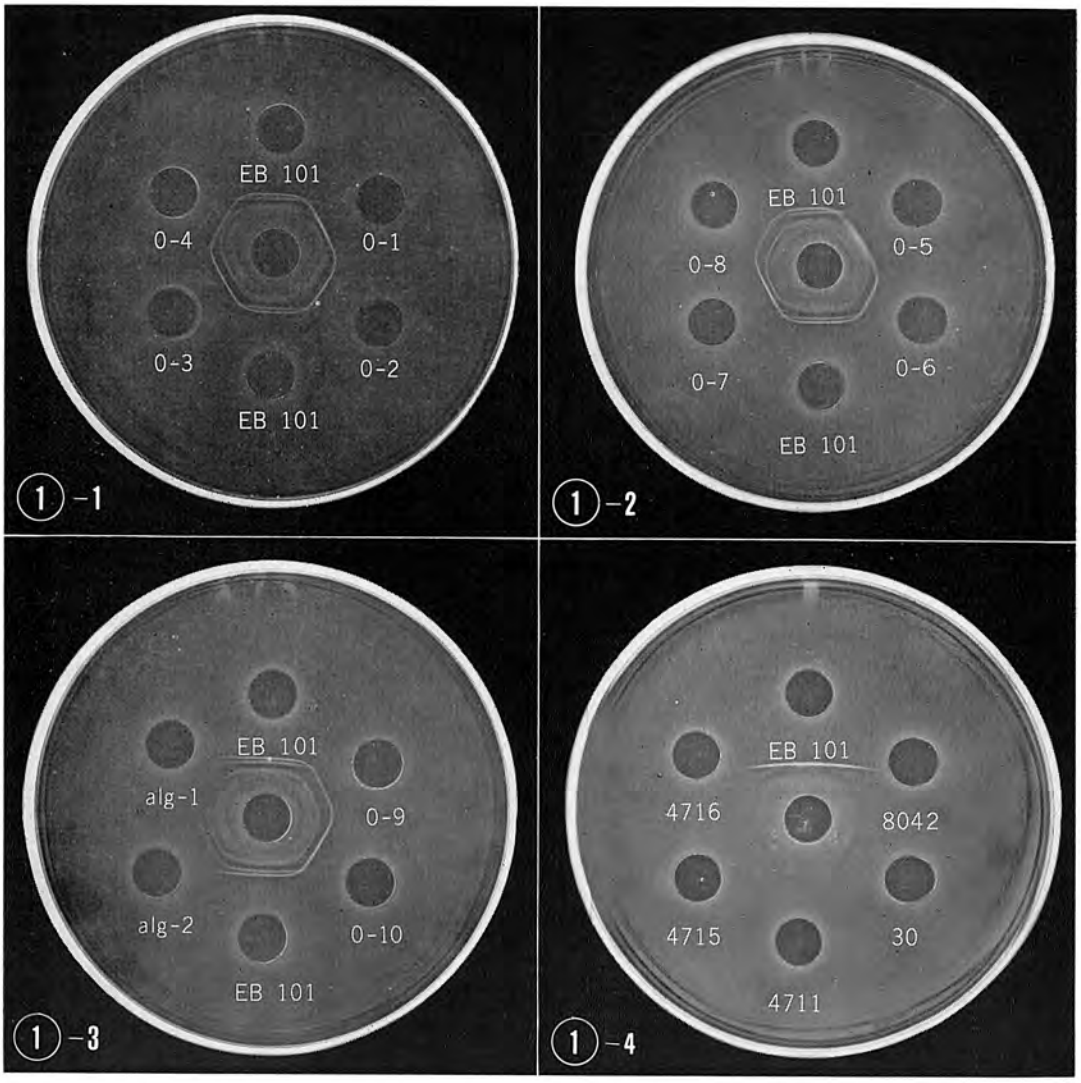


FIGURE 1. *Existence of an antigenic substance common to strains of Vibrio parahaemolyticus.*
The gel deffusion test was carried out as described in the text. Antiserum from Fraction A of V. parahaemolyticus EB 101 was placed in the center well preperations of Fraction A from several strains of V. parahaemolyticus were used as antigens. Symbols indicate the strains from which Fraction A was prepared: EB 101. V. parahaemolyticus EB 101; O-1-O-10; alg-1 and alg-2, V. alginolyticus; 8042, 30,4711, 4715 and 4716, vibrios representing Gardner-Venkatraman's serotypes as described in the text.

was applied on a DEAE-cellulose column and eluted as described in Materials and Methods. Fig. 2 shows a typical pattern of elution from the column. The distribution of A-substance was assayed by the gel diffusion test using an antiserum to Fraction A from *V. parahaemolyticus* O-1. A-substance was found to be eluted with 0.01 M phosphate buffer containing 0.35 M sodium chloride and this fraction was designated as Fraction PPA. It was kept frozen until used. About 10 ml of Fraction PPA, containing about 100 mg of protein per ml were applied on a Sephadex G-200 column and gel filtration was carried out as described Materials and Methods. A typical distribution is shown in Fig. 3. The distribution of A-substance was assayed by the gel diffusion test, as described above, and fraction containing A-substance were collected, concentrated, and purified further.

About 10 mg protein recovered from the sephadex G-200 column were subjected to starch-block electrophoresis as described in Materials and Methods. Fig. 4 shows typical results of this electrophoresis.

A-substance was assayed by the gel diffusion test, collected, concentrated, and resubjected to gel filtration on Sephadex G-200 column.

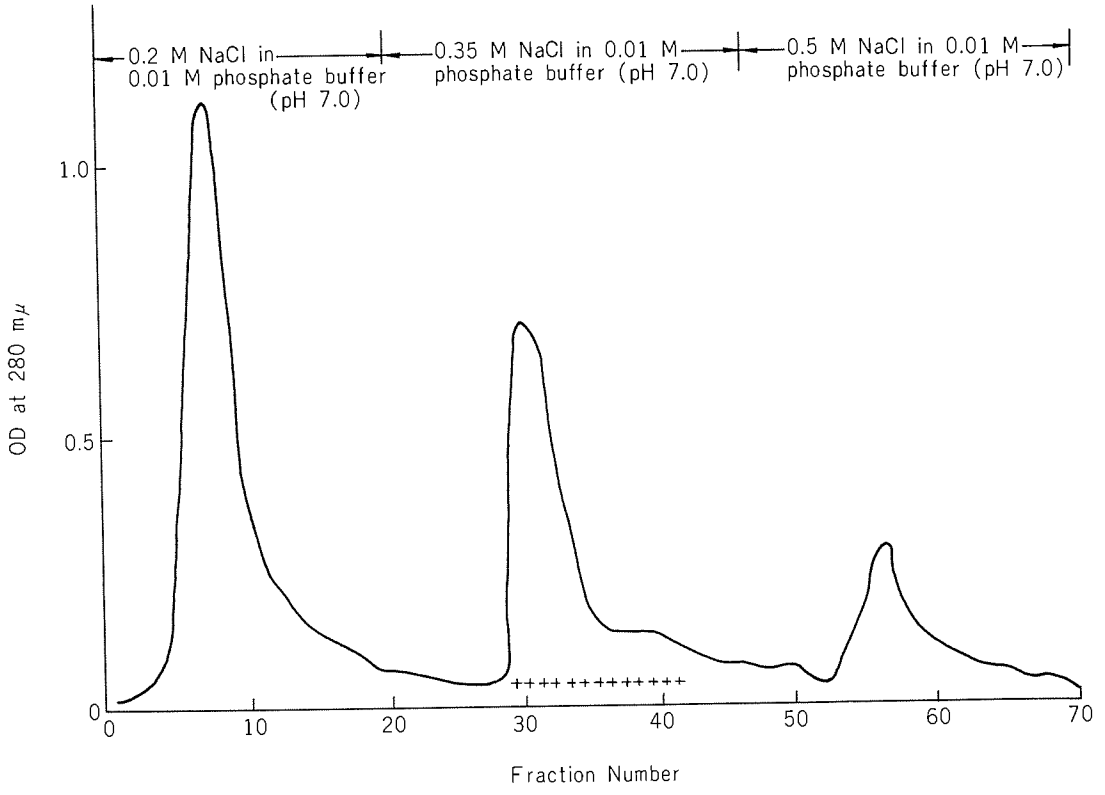


FIGURE 2. *DEAE-cellulose column chromatogram.*
Fraction A of V. parahaemolyticus O-1 was precipitated with 30–45% saturation of ammonium sulfate and dialyzed as described in the text. 500 mg of protein of this fraction were chromatographed on DEAE-cellulose as described in the text. The symbol (+) indicated that A-substance was detected in the fraction by the gel diffusion test.

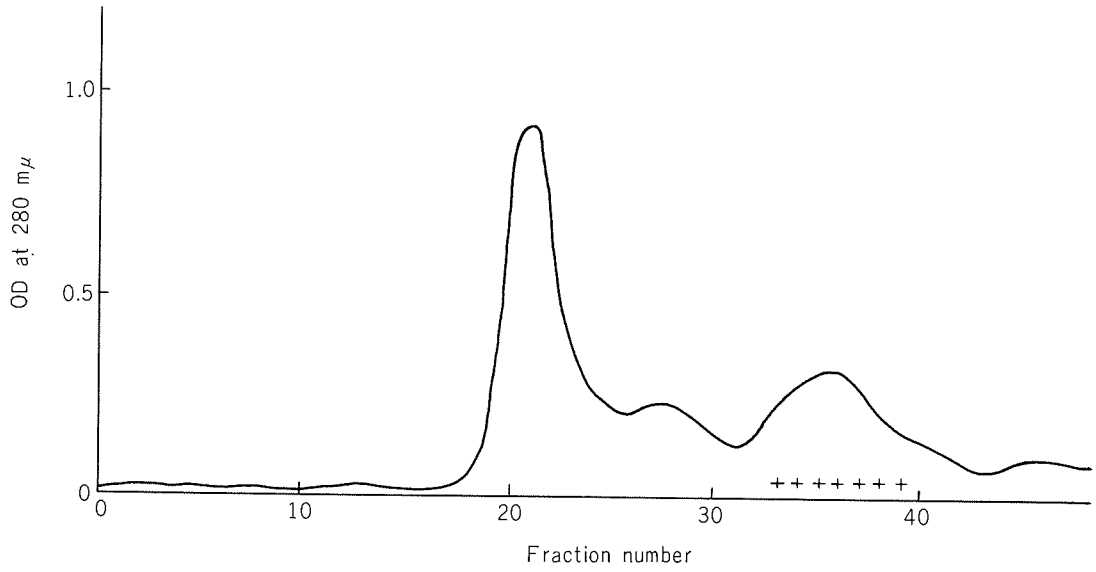


FIGURE 3. *Gel filtration of Fraction PPA on Sephadex G-200 column.*
10 ml of Fraction PPA containing about 100 mg of protein were subjected to gel filtration a Sephadex G–200 column as described in the text. Explanation as in Fig. 2.

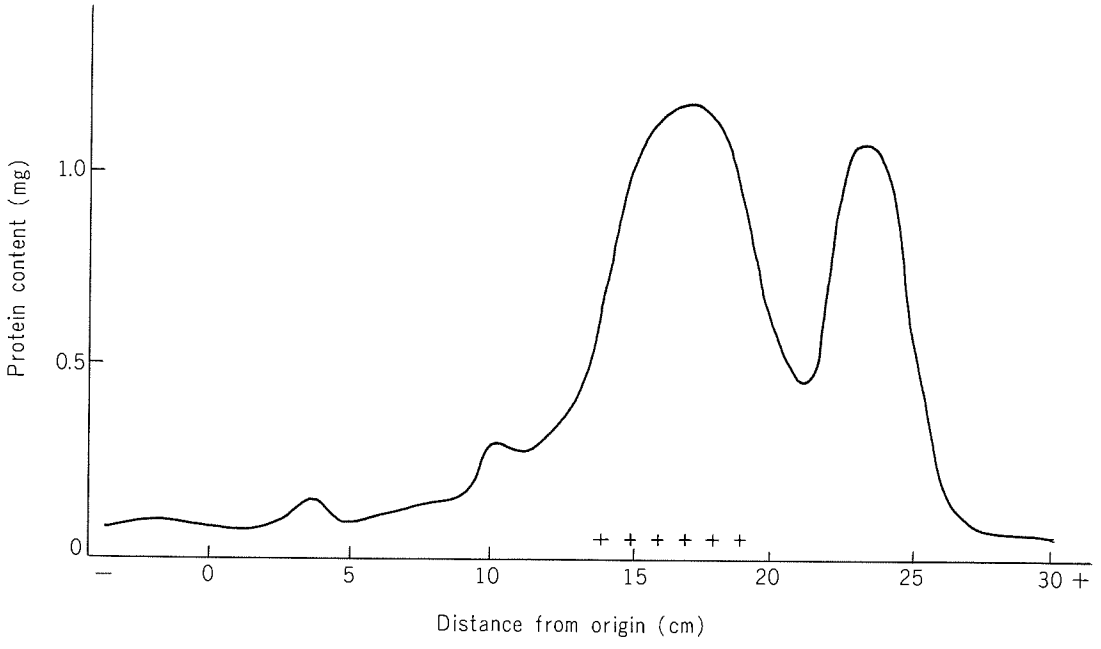
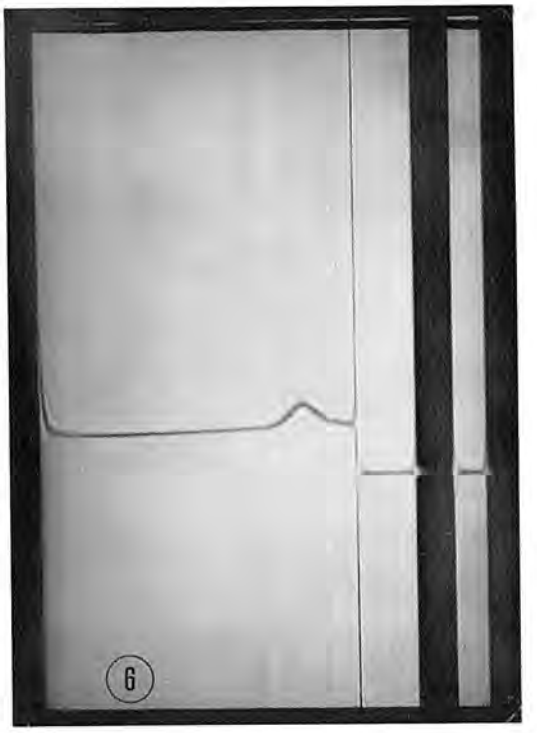


FIGURE 4. *Starch-block electrophoresis of A-substance.*
About 10 mg of protein recovered from the Sephadex G–200 column were subjected to starch-block electrophoresis as described in the text. Explanation as in Fig. 2.

A typical pattern of this gel filtration is illustrated in Fig. 5. A-substance was assayed by gel diffusion test and was collected and used as the purified preparation.

Its purity was examined by analytical centrifugation (Fig. 6), the gel diffusion test (Fig. 7) and immunoelectrophoresis (Fig. 8). In these tests the purified material behaved as a single component.



▶
FIGURE 6 *Analytical centrifugation of purified A-substance.*
Analytical centrifugation was carried out as described in the text. The picture was taken 30 min after reaching maximal speed (55,430 rpm).

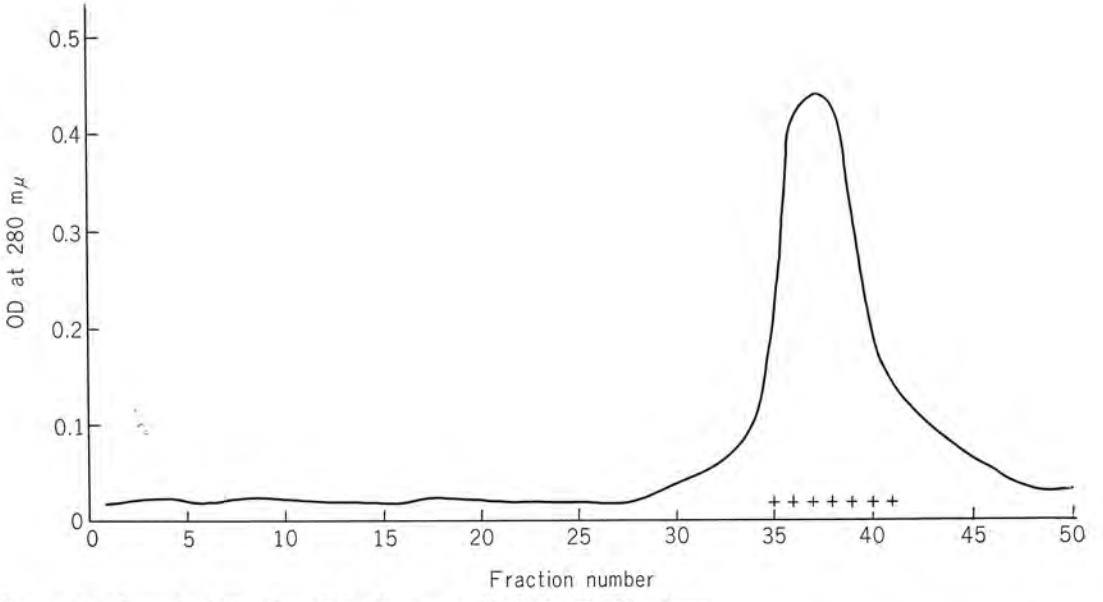


FIGURE 5. *Second gel filtration of A-substance on Sephadex G-200 column.*
A-substance after starch-block electrophoresis was applied on a Sephadex G–200 column. Explanation as in Fig. 3.

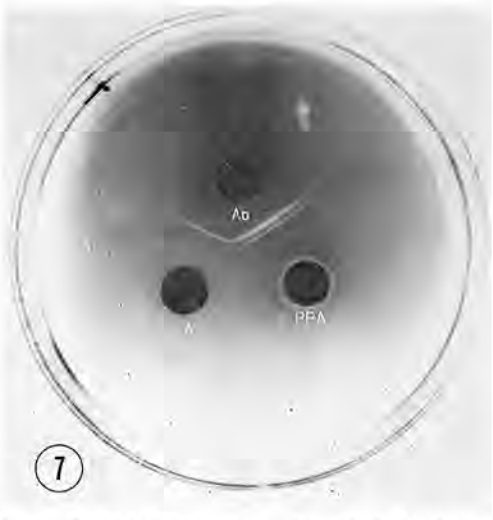


FIGURE 7 *Gel diffusion test of purified A-substance and Fraction PPA.*

The gel diffusion test was carried out as described in the text. Antiserum to Fraction PPA and purified A-substance and Fraction PPA were used as antigens: Ab, antiserum; PPA, Fraction PPA; A, purified A-substance.

FIGURE 8 *Immunoelectrophoresis of purified A-substance.*

Immunoelectrophoresis was carried out as described in the text. Purified A-substance was placed in the hole and antiserum to Fraction PPA was placed in the through.

3. *Distribution of A-substance in strains of V. parahaemolyticus isolated from clinical sources*

To examine the distribution of A-substance in different strains of *V. parahaemolyticus*, crude antigens were prepared as described in Materials and Methods from 84 strains isolated at the Public Health Research Institute of Kobe City and from 42 strains isolated at Momoyama Osaka Municipal Hospital. Gel diffusion tests were made using these antigens and the antiserum to A-substance and some of the results are shown in Fig. 9. Antigens from all the strains of *V. parahaemolyticus* tested gave a common precipitation line but that from *V. alginolyticus* did not.

FIGURE 9. *Existence of A-substance in crude antigens of V. parahaemolyticus.*

The gel diffusion test was carried out as described in the text. Antiserum to purified A-substance of V. parahaemolyticus O–1 was placed in the center well and crude antigens prepared from several strains of V. parahaemolyticus and V. alginolyticus were placed in side wells as indicated: 1, V. parahaemolyticus KB 67–3013 (O-N1); 2, V. parahaemolyticus KB 67–3022 (O–3); 3, V. parahaemolyticus MY 67–029 (O–4); 4, V. parahaemolyticus MY 67–003 (O–2); 5, 6 and 7, V. alginolyticus; 8, purified A-substance from V. parahaemolyticus O–1.

4. *Evidence that A-substance is different from O-, K-, and flagellar-antigens*

The above results demonstrated that A-substance is an antigen common to strains of *Vibrio parahaemolyticus* which is not present in other vibrios. As *V. parahaemolyticus* has three antigenic components (O-, K-, and flagellar-antigens), the relationship of A-substance to these antigens was studied.

If A-substance is the same antigenic substance as O-antigens, a common precipitation line should not be obtained when crude antigens with different O-antigens are tested with the antiserum to purified A-substance. Similarly if A-substance is the same antigenic substance as K-antigens, no common precipitation line will be observed in the gel diffusion test using crude antigens having different K-antigens and the antiserum to purified A-sub-

stance. Results of an experiment on this are shown in Fig. 10. In this experiment, crude antigens from strains with different serotypes (O2: K2, O3: K4, O4: K8, O5: K14, O6: K18, O7: K19, O8: K20, O9: K23, O10: K24, ON1: K36, ON2: K unknown) were prepared and subjected to gel diffusion with the antiserum to purified A-substance. As expected, a common precipitation line was observed as shown in Fig. 10. This indicates that A-substance was different from O- and K-antigens. This was confirmed using the gel diffusion test for A-substance and antisera to O- and K-antigens. A-substance did not give any precipitation lines with the antiserum to either O- or K-antigen.

To study the relationship of A-substance to flagellar-antigen, flagella of *V. parahaemolyticus* O-1 were purified as described previously (Shinoda et al., 1968) and antiserum to purified flagella was prepared. On immunoelectrophoresis, A-substance of *V. parahaemolyticus* O-1 did not give any precipitation lines with the antiserum to purified flagella of *V. parahaemolyticus* O-1 and *vice versa*. This indicated

FIGURE 10. *Gel diffusion test of purified A-substance with crude antigens having different O- and K-antigens.*

Antiserum to purified A-substance was placed in wells 1 and 2 and purified A-substance in well 3. Crude antigens from different strains were prepared and placed in wells 4–14. Their antigenic structures (O- and K-antigen) were as follows: 4, 02: K2; 5,03: K4; 6,04: K8; 7,05: K14; 8,06: K18; 9,07: K19; 10,08: K20; 11,09: K23; 12,010: K24; 13,0N1: K36; 14,0N2: K unknown.

that purified A-substance and purified flagella do not share any common antigenic substances.

DISCUSSION

An antigenic substance (A-substance) was isolated from *Vibrio parahaemolyticus* and demonstrated to be a common antigen of this organism. Using the gel diffusion test, it was found that A-substance was present in all 126 strains of *V. parahaemolyticus* isolated from clinical sources. These results suggest that it is possible to use the antiserum to purified A-substance for identification of *V. parahaemolyticus*.

Evidence that A-substance differs from O-, K- and flagellar-antigen is presented here, but further study is necessary to elucidate the relationship of A-substance to these antigenic components. It was recently suggested that O-antigen of *V. parahaemolyticus* was a lipopolysaccharide (Torii et al., 1969). As A-substance has been found to be a protein (Miwatani, Shinoda and Fujino; unpublished observation), O-antigen and A-substance may be different antigenic substances. Omori et al. (1966) reported on the chemical nature of K-antigen which is also not a protein and seems to be different from A-substance. However, A-substance may be a part of these antigenic substances, with a protein nature and may be common to all strains.

We found by immunoelectrophoresis that purified flagella did not give any precipitation lines with the ansiserum to purified A-substance. This suggests that the antigens of A-substance and flagella are not the same. However, in contrast to the findings of Terada (1968) and Sakazaki et al. (1968), purified flagella do not have an antigen in common with *V. parahaemolyticus*, according to the results of immuno-immobilization test (Miwatani, Shinoda and Fujino; unpublished observation). On purification flagella may lose some antigenic substances, which are common to this organism. This possibility is now under investigation.

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