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ENZYME DEGRADING *VIBRIO PARAHAEMOLYTICUS* K15-ANTIGEN[1]

KOICHI KURODA and GENDO OMORI

Osaka City Institute of Hygiene, Kitaogimachi, Kita-ku, Osaka

MITSUO TORII[2]

Departmemt of Immunology

Research Institute for Microbial Diseases, Osaka University, Yamada-Kami, Suita, Osaka

KUNIO IMAMURA

Research Foundation for Microbial Diseases of Osaka University, Yamada-Kami, Suita, Osaka

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SUMMARY The K-antigen of *Vibrio parahaemolyticus* A55 (O5 : K15) is degraded by an enzyme produced by a microorganism isolated from soil which seems to be a new species of the genus *Bacillus*.

The enzyme preparation was obtained from the supernatant fluid of a culture of this organism. Incubation of the enzyme with K-antigen resulted in loss of the ability of the antigen to precipitate homologous antisera and in release of reducing sugar and an acidic N-acetyl hexosamine. Some of the degradation products inhibited the precipitin reaction.

The enzyme also acted on living cells of *V. parahaemolyticus* enhancing their agglutination by O-antiserum.

INTRODUCTION

Capsular K-antigen of *Vibrio parahaemolyticus* A55 (O5 : K15) was isolated by Omori et al. (1966) and its purification and properties have been described in detail (Omori et al., 1966). Chemical analyses of the antigen which was an acidic polysaccharide showed that it contained hexosamine 17–22%, reducing sugar 15–20%, protein 0.4–1.7% and acetyl 14.5–15.7%. However, neither a known neutral sugar nor hexosamine has yet been detected in hydrolysates of the antigen. Moreover, in spite of its acidic nature no uronic acid, sialic acid or phosphoric acid was found in it.

It seemed likely that the antigen would be rather labile in mineral acid, so attempts were made to isolate an enzyme capable of hydrolyzing the antigen. Samples of soil from various areas were added to medium containing K-antigen and the presence of antigen in the culture medium was tested. An organism producing an enzyme which degraded the antigen was found in a soil sample obtained in Gifu prefecture, Japan. This paper describes the isolation of this organism, preparation of

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2 To whom correspondance should be addressed.

enzyme from it and degradation of K-antigen with the enzyme.

MATERIALS AND METHODS

1. *Media for the bacteria*

The isolated bacteria did not grow in ordinary nutrient broth, so a medium (pH 7.2) containing crude K15-antigen 0.01%, tryptone (Difco) 1%, yeast extract (Difco) 0.5% and sodium chloride 0.5% was used. It was autoclaved at 121 C for 20 minutes. For preservation of the bacteria, 1.2% agar was added to the medium. The bacteria were also grown on trypticase soy agar (BBL).

2. *K15-antigen of Vibrio parahaemolyticus A55 (O5: K15)*

V. parahaemolyticus A55 (O5 : K15) was grown at 37 C overnight on agar medium (pH 7.2), containing polypeptone (Daigo) 1%, meat extract (Wako) 0.5% and sodium chloride 3%. Then the cells were harvested, washed with 3% sodium chloride, and heated at 100 C for 2 hours in 3% sodium chloride. The mixture was centrifuged and the supernatant was dialyzed against M/50 phosphate buffer, pH 7.2. The mixture inside the tubing was digested with 50 μg/ml of ribonuclease (Worthington), at 37 C for 3 hours and then treated with an equal volume of 90% phenol at 60 C for 30 minutes with stirring as descrived by Westphal et al. (1952). After cooling, the mixture was centrifuged and the aqueous layer was dialyzed against distilled water and lyophilized. This was a crude antigen preparation. The crude antigen was purified by chromatography on a DEAE-cellulose column twice as reported by Omori et al. (1966).

3. *Antisera*

K-antisera (serum No. 3 and 6) were prepared by injecting cells of *V. parahaemolyticus* A55 which had been killed with formalin into rabbits, as described by Omori et al. (1966). Their agglutinin titers against homologus living cells were 1 : 6400.

O-antiserum (serum No. 5) was prepared with heat killed organisms as described by Omori et al. (1966). The O-agglutinin titer of the antiserum was 1 : 800.

4. *Serological methods*

The precipitin test was performed by both the capillary tube method and Ouchterlony's technique (Ouchterlony, 1949).

Quantitative assay of inhibition was carried out by the method described by Kabat (1961) as follows:

0.2 ml of antiserum (serum No. 3) was incubated with 0.2 ml of test sample at 37 C for 30 minutes. Then 0.2 ml of antigen was added and the mixture was incubated at 37 C for 1 hour and then kept at 4 C for 2 days. The quantity of antigen used was equivalent to the amount of precipitating antibodies. Precipitates were washed twice with chilled physiological saline and dissolved in 1N sodium hydroxide. Precipitated protein was determined by the Folin-Lowry method (Lowry et al., 1951).

O-agglutination was performed by mixing 0.45 ml of cell suspension with 0.05 ml volumes of serially diluted antiserum and incubating the mixtures at 50 C overnight. Agglutination was performed in 3% sodium chloride solution (Sakazaki, 1967).

5. *Analytical methods*

N-acetyl hexosamine was determined by Morgan-Elson's method (Reissig et al. 1955). Reducing sugar was estimated by Somogyi-Nelson's method (Somogyi, 1952). Turbidity of cultures was measured in a Shimadzu spectrophotometer at 600 mμ. Paper electrophoresis was performed in a Model C-1 apparatus (Toyo Kagaku) using Toyo filter paper No. 51 and M/10 acetate buffer, pH 7.0. Sugars and N-acetyl hexosamines on paper were detected by the silver nitrate method (Trevelyan et al. 1950) and Morgan-Elson's method (Partridge, 1948), respectively.

RESULTS

1. *Isolation of microorganism*

Samples (1.0 g) of soil were added to 1.0 ml of phosphate buffer, pH 7.0, containing 0.5% sodium chloride, and the supernatants (0.1 ml) of the mixtures were incubated with 1.0 ml of 1% crude K15-antigen solution at 37 C. The culture supernatants were tested daily for the presence of antigen using the precipitin reaction. One culture derived from farm soil eliminated the serological activity from the supernatant fluid after 2 weeks.

It was very hard to isolate the microorganism which produced the enzyme degrading the K-antigen, because contaminating bacteria always grew very fast. However, eventually

it was found that on heating the culture at 80 C for 10 minutes the contaminating bacteria were completely eliminated and a pure culture was obtained. The heated culture was streaked onto an agar plate and incubated at 37 C. Very fine colonies appeared after 24–48 hours. After 72 hours the colonies appeared as white dots covered with somewhat transparent growth.

2. *Characteristics of the organism*

The organisms are aerobic rods with round ends and are gram positive in young cultures. They produce oval endo-spores which are resistant to heating at 80 C for 10 minutes. Optimum growth was obtained at 33 C but there was some growth at both 37 C and 25 C. The organisms produced catalase.

As the bacteria seemed to belong to the genus *Bacillus*, efforts to identify them were made following the description of Smith et al. (1952), Breed et al. (1957) and Wolf and Barker (1968). The results were as follows: fermentation of glucose, positive; fermentation of arabinose, xylose and mannitol, negative; hydrolysis of starch, casein and gelatin, negative; no growth at 65 C and pH 7.0 or at 60 C and pH 6.0 in the growth medium; no growth in trypticase soy broth medium containing 4, 7, or 10% sodium chloride; production of indol, negative; reduction of nitrate, slight; oxidase, positive. These biological behaviors indicated that this microorganism is not identical with any of the species of *Bacillus* described by Smith et al. (1952), Breed et al. (1957) or Wolf and Barker (1968).

3. *Relationship between growth and enzyme activity*

The organisms were cultured at 33 C in the medium containing K-antigen with shaking. At intervals, the turbidity of the culture and the activity of the enzyme degrading antigen were measured. The enzyme was assayed as follows: the culture was centrifuged and an aliquot of the supernatant was diluted with 3 volumes of M/50 Tris buffer, pH 7.8, and then mixed with an equal volume of K15-antigen solution (final concentration: 0.05%). After addition of 1 drop of toluene, the mixture was incubated at 37 C for 1 hour and then the concentration of N-acetyl hexosamine was determined. Fig. 1 shows that the enzyme activity increased with growth of the cells and reached a maximum level after 13 hours, at the time when full growth of the organisms was obtained.

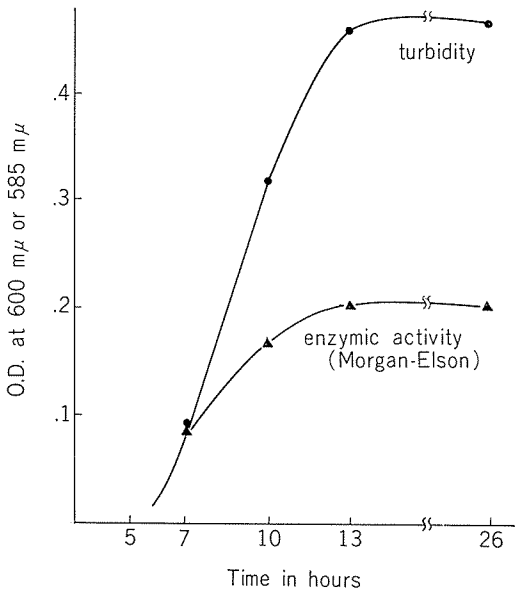

FIGURE 1. *Relationship between growth of cells and enzymic activity.*

Turbidity was read against liquid medium.

O.D. for enzyme assay was corrected by subtraction of the value for the culture supernatant control.

O.D. at 600 mμ is for turbidity and that at 585 mμ is for enzyme activity.

4. *Preparation of enzyme*

A fully grown culture was centrifuged and ammonium sulfate was added to the supernatant to 80% saturation. The mixture was stood at 4 C overnight, and then centrifuged. The supernatant and precipitate were each dialyzed against distilled water and concentrated. The bacterial cells were suspended in M/50 Tris buffer, pH 7.8, and disrupted by

sonication (10 KC. for 7 min).

The enzyme activities of these three fractions were determined and results are shown in Table 1. The highest activity was found in the precipitate, so this fraction was lyophilized and used for experiments.

TABEL 1. *Enzyme activities of fractions of bacterial culture*

Fraction	volume ml	Activity ml [a]
Whole culture	70	64
Bacterial cells	50	64
Precipitate	7	640
Supernatant	4	8

a The activity shows the highest dilution at which each fraction affected the precipitating ability with antiserum of K-antigen in agar. The experimental conditions were as follows: fractions (0.5 ml) were serially diluted with Tris buffer and mixed with 0.05% antigen solution (0.5 ml) and 1 drop of toluene. After incubation at 37 C overnight, each mixture were heated at 100 C for 10 minutes and tested for its precipitin reaction with K15-antiserum (serum No. 6).

5. *Effect of the enzyme on release of acetyl hexosamine from K15-antigen*

The effect of the enzyme on K15-antigen was measured by determination of N-acetyl hexosamine. The enzyme (2 mg/ml of water) was mixed with equal volumes of antigen solution (final concentration 0.05%) and M/50 Tris buffer, pH 7.8. After addition of 1 drop of toluene, the mixture was incubated at 37 C. At intervals, aliquots were taken out and heated and their N-acetyl hexosamine content was determined. As shown in Fig. 2, N-acetyl hexosamine was released by the enzyme, and the release reached 10% of the total as N-acetyl glucosamine after 22 hours.

6. *Effect of the enzyme on release of reducing sugar from K-antigen*

Released sugar was estimated after similar treatment of K-15 antigen with the enzyme. Fig. 2 shows that reducing sugar was also liberated by the enzyme, the maximum amount being 27% of the total as glucose after 22 hours.

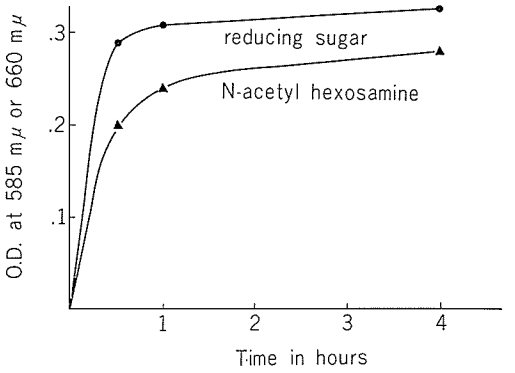

FIGURE 2. *Release of reducing sugar and N-acetyl hexosamine from K15-antigen.*
O.D. at 585 mμ is for N-acetyl hexosamine and that at 660 mμ is for reducing sugar.

7. *Effect of the enzyme on loss of precipitating ability of the antigen and inhibition of precipitation by the product*

K15-antigen was treated with the enzyme as described above. Aliquots were taken at intervals and tested by the precipitin reaction. Fig. 3 shows that the intensity of the precipitin line decreased with increase in the incubation time and after 24 hours incubation the ability of the antigen to cause precipitation in agar was completely lost. The product evidently inhibited the precipitin reaction.

8. *Effect of pH on enzyme activity*

Enzyme activity was estimated at various pH values. The buffers used were M/50 Tris (at and above pH 7.4) and M/50 phosphate (pH 7.0). Enzyme solution (2mg/ml of water) was incubated with equal volumes of buffer and K-antigen solution (final concentration: 0.05%) at 37 C for 1 hour. Aliquots were taken to measure the concentration of reducing sugar. Fig. 4 shows that the optimum pH was 7.8.

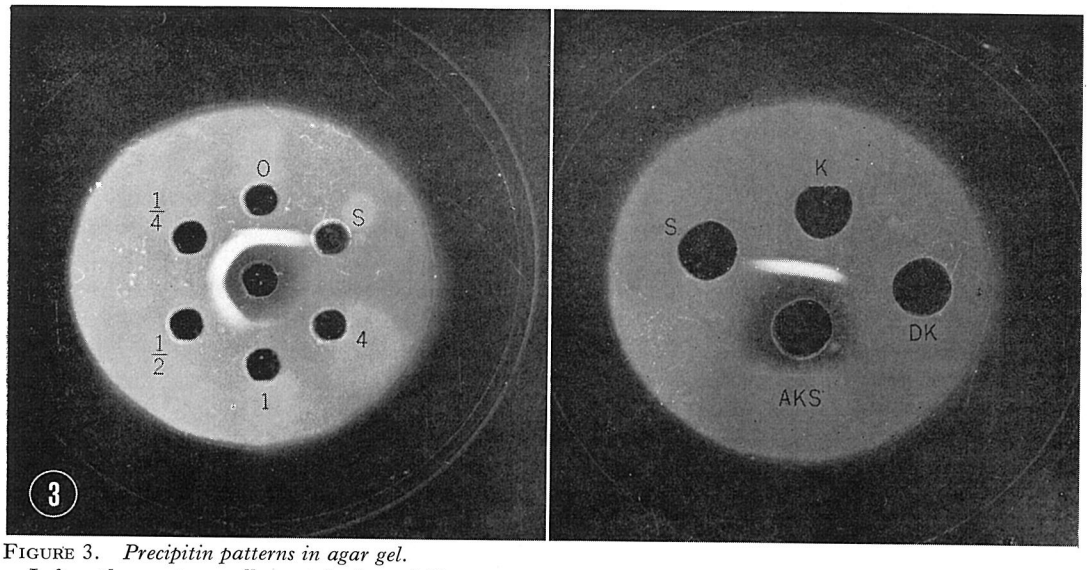

FIGURE 3. *Precipitin patterns in agar gel.*

Left: the center well contained anti-K serum (No. 6); 0, 1/4, 1/2, 1, and 4, K-antigen incubated with the enzyme for 0, 1/4, 1/2, 1, and 4 hours, respectively; S, saline.

Right: K, K-antigen; DK, K-antigen incubated with the enzyme for 24 hours; AKS, anti-K serum (No. 6); S, saline.

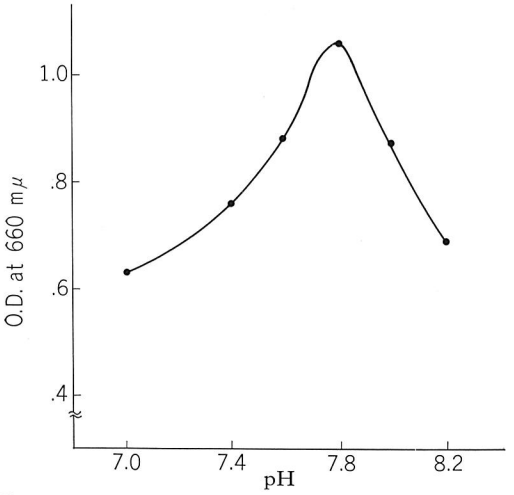

FIGURE 4. *Effect of pH on enzymic activity.*

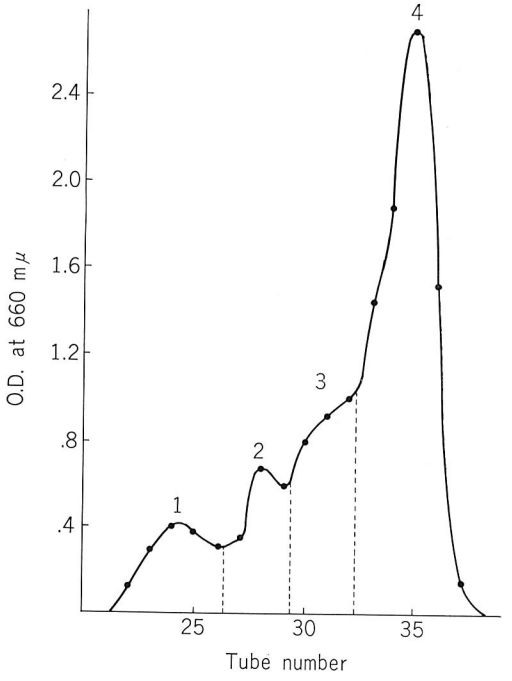

FIGURE 5. *Chromatography of degraded K-antigen on Sephadex G-15.*
Sample: 0.4 ml.
column size: 1.4×90 cm.
flow rate: 6 ml/hr.
fraction volume: 2 ml.
estimation: reducing sugar.

9. *Partial fractionation of degradation products*

Six hundred mg of K-antigen and 200 mg of enzyme were dissolved in 100 ml of M/50 Tris buffer, pH 7.8, and a small quantity of toluene

was added. The mixture was incubated at 37 C for 24 hours and then filtered through a Sartorius collodion bag to obtain products of low molecular weight. The filtrate was mixed with 9 volumes of ethanol (95%) and kept at −20 C overnight. The resulting precipitate was removed by centrifugation, and the supernatant was concentrated and subjected to Sephadex G-15 gel filtration. The result in Fig. 5 shows that four fractions were obtained. The characteristics of the fractions are presented in Table 2. All fractions gave a positive Morgan-Elson reaction and a negative ninhydrin reaction and moved to anode on paper electrophoresis at pH 7.0. The spots developed with silver nitrate reagent and Morgan-Elson reagent coincided completely. All fractions more or less inhibited the precipitin reaction.

10. *Effect of the enzyme on living bacteria*

V. parahaemolyticus is a halophilic microorganism, so it is usually cultured in media containing 3% sodium chloride. On the other hand, enzyme activity was low in the presence of a high salt concentration, as shown in Fig. 6. Accordingly organisms were grown in the usual medium containing 3% sodium chloride. Then they were washed with 3% sodium chloride solution and suspended in M/50 Tris buffer containing 1% sodium chloride. The suspension was incubated with the enzyme (final concentration of enzyme, 3.5 mg/ml) at 37 C. Aliquots were taken at intervals and the organisms were washed twice with 3% saline and subjected to the O-agglutination test. As shown in Table 3, O-agglutination was enhanced by the action of the enzyme and the

TABLE 2. *Characteristics of the four fractions obtained by Sephadex gel filtration*

Fractions	1	2	3	4
Morgan-Elson reaction	+	+	+	+
Ninhydrin reaction	−	−	−	−
Direction of movement on paper electrophoresis[a]	anode	anode	anode	anode
Inhibition of precipitation[b]	13%	10%	24%	25%

a Spots were detected with silver nitrate-sodium hydroxide reagent and Morgan-Elson reagent.
b These inhibitions were obtained with 120 μg, 92 μg, 390 μg or 440 μg (as N-acetyl glucosamine), respectively, of the fractions. 1300 μg of protein per tube was precipitated when inhibitor was not added.

TABLE 3. *O-agglutination test of V. parahaemolyticus treated with the enzyme degrading K-antigen*

Treatment for	anti O-serum (serum No. 5)						
	×40[a]	×80	×160	×320	×640	×1280	×2560
2 hr	+[b]	+	+	−[c]	−	−	−
control	−	−	−	−	−	−	−
6 hr	+	+	+	+	+	−	−
control	−	−	−	−	−	−	−
22 hr	+	+	+	+	+	−	−
control	−	−	−	−	−	−	−
2 hr boiled	+	+	+	+	+	−	−

a Serum dilution. *b* Positive agglutination. *c* No agglutination.

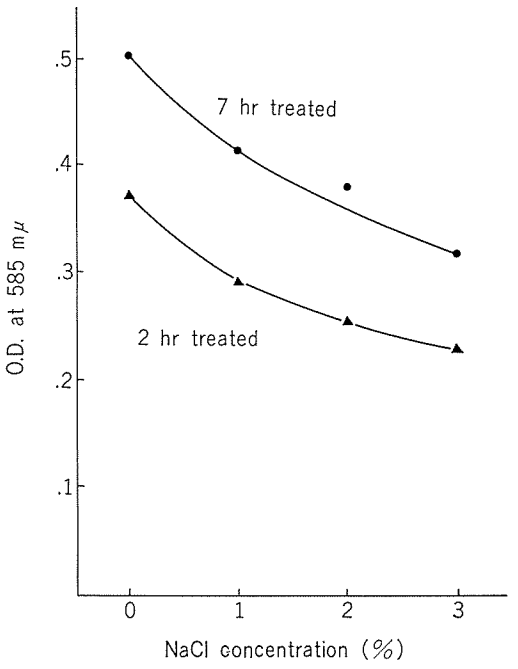

FIGURE 6. *Relationship between concentration of sodium chloride and enzymic activity.*
Enzymic activity was measured by the Morgan-Elson reaction.
Buffer: Tris M/50, pH 7.8
K-antigen: 250 μg/ml
Enzyme: 2.5 mg/ml

agglutination of cells after 6 or 22 hours incubation was the same as that of cells after two hours heat treatment.

DISCUSSION

Microorganisms which produced specific enzymes capable of degrading antigens have been reported by many authors. Thus, Dubos and Avery (1931), in their pionier work of this kind, isolated a bacterium from soil which produced an enzyme hydrolyzing type III pneumococcal polysaccharide. Landsteiner and Chase (1935) used several organisms for degradation of the blood group A substance. Iseki and Furukawa (1959) isolated enzymes from *Clostridium tertium*, *Clostridium maebashi* and *Bacillus fulminans* capable of acting on blood group A, B and H substances, respectively. Gadebusch and Johnson (1960, 1961) obtained *Alkaligenes* sp. which specifically degraded *Cryptococcus neoformans* polysaccharide. Kotani et al. (1959) isolated three strains from soil which lyse the cell walls of BCG, *Corynebacterium diphtheriae*, *Staphylococcus aureus* and *Streptococcus pyogenes*. McCarty (1952) separated an enzyme from a culture of *Streptomyces* which acted on streptococcal cell wall polysaccharide. Recently, Baker and Whiteside (1965) obtained an enzyme degrading Vi-antigen from *Bacillus sphaericus*.

The present organism also produced a particular enzyme capable of degrading K-antigen of *V. parahaemolyticus*. This organism seems to be a new species of the genus *Bacillus* from its biological properties, but further investigations are required to confirm this. It is tentatively proposed to refer to this organism as Bacillus species RIMD 1001.

The enzyme activity was mostly found in a fraction precipitated by ammonium sulfate, but the activity in the cells was also fairly high. It will be interesting to see whether the intra and extracellular enzymes are the same.

A positive reaction with Morgan-Elson reagent indicates that the sugars contain C-1 unsubstituted, C-2 with an acetyl amino group and C-4 hydroxyl free (cf. Horton, 1969). So, the paralle rates of liberation of reducing sugars and N-acetyl hexosamines from the present K-antigen by the enzyme seem reasonable, because only hexosamine was found as a major sugar constituent of the antigen (Omori et al. 1966). The maximal amounts of release of N-acetyl hexosamine and reducing sugar were 10% and 22%, respectively, of the total amounts, even after 22 hours incubation. This low value may be due partly to incomplete hydrolysis by the enzyme, and partly to the weak colour reactions of the products.

The fact that the enzyme caused loss of the ability of the antigen to precipitate with antiserum and that the degradation products

inhibited the precipitin reaction suggests that the enzyme probably caused simple fragmentation of the antigen molecule. Enzymes degrading Vi-antigen caused so much decomposition that the products did not inhibit precipitation at all (Baker and Whiteside, 1965).

The four fractions of degradation products obtained all gave a positive Morgan-Elson reaction, so these products may be 2-acetylamino 2-deoxy sugars or their derivatives or oligomers. All the fractions moved to the anode on paper electrophoresis, so they must all have some acidic group such as a carboxyl group but at present it is uncertain whether they contain an acetyl aminohexuronic acid residue. Aminohexuronic acids have been found in several bacteria (Heyns et al. 1959; Williamson and Zamenhof, 1963; Perkins, 1963; Hanessian and Haskell, 1964; Smith, 1968; Mayer, 1969) and are reported to be very unstable on acid hydrolysis (Perkins, 1963; Hanessian and Haskell, 1964). This antigen might also contain an aminohexuronic acid, because on its acid hydrolysis no known sugar has yet been identified (Omori et al., 1966). The antigen requires more careful chemical analysis.

Table 3 shows that the enzyme acted on living cells of *Vibrio parahaemolyticus* enhancing their O-agglutination. This suggests that the enzyme acted on the capsule of the bacteria as well as on isolated K-antigen and probably removed the antigen, exposing the O-antigen site to the outer side.

Further purification of the enzyme and further studies on the mechanism of the enzyme action and the nature of the degradation products are in progress.

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