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Title: PARTIAL PURIFICATION AND CHARACTERIZATION OF HEMOLYSIN FROM A PSYCHROTROPHIC KANAGAWA POSITIVE MARINE VIBRIO

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Psychrotrophic Kanagawa positive marine vibrios distinct from V. parahaemolyticus were isolated from shellfish collected in Yaquina Bay, Oregon. Sample enrichment in GSTB was followed by culture isolation on TCBS agar. The 235 vibrio isolates obtained were screened for gram reaction and morphology, Kanagawa reaction on Wagatsuma agar and their response to selected biochemical tests. Approximately 11% of the isolates were Kanagawa positive. Isolate 123-S1 was selected for further study based on the production of a high level of hemolysin and other pertinent characteristics.

The organism was grown in Lib-X broth and the hemolysin was precipitated from a cleared supernatant with a 55% saturation of ammonium sulfate. The hemolytic substance was partially purified by DEAE-cellulose chromatography and

Sephadex G-100 gel filtration. The hemolysin contained protein that was essential for activity, was thermolabile, and was more active against rabbit erythrocytes at 37°C than at lower temperatures. The molecular weight was estimated at 55,000 by Sephadex G-100 gel filtration. Hemolytic activity was partially inactivated by gangliosides and was lowered against horse erythrocytes. The hemolysin did not precipitate, by Ouchterlony diffusion, with antibody prepared against vibriolysin from V. parahaemolyticus WP-1. Amino acid analysis showed the hemolysin was high in aspartic and glutamic acids and low in arginine and histidine. Electrophoresis on a SDS polyacrylamide gel revealed 3 major bands.

The hemolysin of 123-S1 and the hemolytic exotoxin of V. parahaemolyticus had some similar and dissimilar characteristics. A possible significance of these results is that Vibrio spp. other than V. parahaemolyticus might serve as the reservoir for the Kanagawa phenotype. The possibility that the genetic determinant for the production of toxin and pathogenicity might be transmitted by bacteriophage or plasmids among Vibrio spp. in the marine environment is discussed.

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Typed by Deanna L. Cramer for Susan Peters Huggins

In memory of Andy

- who probably would have been pleased about this

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INTRODUCTION

The idea that some pathogenic bacteria produce harmful, diffusible compounds, or exotoxins, was introduced early in the history of microbiology. Bacterial toxins are generally proteins or contain substantial amounts of polypeptides. However, the chemical characterization of most toxins is incomplete and the mechanisms of pathogenicity are understood in only a few cases.

Toxins vary widely in biological potency and in the type of harmful effect produced. There is no consensus regarding the degree of toxicity that any factor must possess to be considered a bacterial toxin (2).

Many toxins have a cytolytic effect on a variety of mammalian cells. Toxins that lyse erythrocytes are also hemolysins. However, the actions of most are not confined exclusively to erythrocytes and thus the term hemolysin is too restrictive. The hemolytic substance produced by Vibrio parahaemolyticus is an example of a toxin that produces cytolytic effects on several types of mammalian cells.

V. parahaemolyticus is a slightly halophilic marine bacterium and some strains may be pathogenic. Pathogenic strains of V. parahaemolyticus differ from the non-

pathogenic strains by their ability to produce a beta-hemolysis (the Kanagawa phenomena) on Wagatsuma agar, a special salt blood agar medium. Pathogenic strains are capable of causing severe gastroenteritis and secondary wound infections.

The ecological or molecular basis for toxin production is not understood. It is not known why only some strains are able to produce toxin and why it is often a transient characteristic. It has been suggested that the genes for toxin production in V. parahaemolyticus may be extrachromosomal and acquired from genetic exchange (J. A. Baross, Ph.D. dissertation, University of Washington, Seattle, 1972; 3, 7, 8, 18).

The presence of beta-hemolytic marine vibrios, that are biochemically different from V. parahaemolyticus have been briefly mentioned (5, 55). However, there is no pertinent information available on these unclassified vibrios. The possibility that a marine vibrio might act as the genetic reservoir for the production of hemolytic toxin in V. parahaemolyticus has not been seriously considered.

The object of this research was to isolate Kanagawa positive pyschrotrophic vibrios from a marine environment and partially purify a hemolysin to determine its chemical and physical characteristics. These characteristics would then be compared to known properties of exotoxin from V. parahaemolyticus. The information obtained might help to

determine the importance of Kanagawa positive psychrotrophic marine vibrios and their possible role as the genetic reservoir for the Kanagawa phenotype.

LITERATURE REVIEW

Although Takikawa (15) was credited with noting the medical importance of V. parahaemolyticus, and Fujino (55) described its ability to produce hemolysins, the association of Kanagawa type hemolysin with pathogenicity was first observed by Kato (50). Using a salt blood agar medium, modified Wagatsuma agar, Sakazaki (38) demonstrated that 96.5% of 2700 cultures isolated from fecal specimens of patients suffering from gastroenteritis were hemolytic. But only 1% of 650 environmental isolates were hemolytic on the same medium. Later others, especially, Miyamoto (33), emphasized this relationship between the source of the isolate and its hemolytic activity.

The hemolytic reaction observed with V. parahaemolyticus results from at least two processes. One is the attachment and penetration of erythrocytes and the other is the fading color of released hemoglobin. The former is caused by the hemolysin and the latter is the result of several factors such as pH, nitrite and glutathione content of the medium (16).

The type of carbohydrate used, the sodium chloride concentration and the final pH of Wagatsuma agar affect the resultant hemolytic reaction (14). It was shown that the addition of 0.5% mannitol to the medium gave the best results when known Kanagawa positive and negative strains

were tested. Wagatsuma agar supported the production of hemolysin if 7% sodium chloride was added. This high salt concentration appears to influence the diffusibility of hemolysin. The final pH (8.0) of the medium was also important (14).

A partial purification of the substance (55) was attempted in an effort to understand the importance of the hemolysin produced by V. parahaemolyticus and its effects on red blood cells. Zen-Yoji (55) showed that the hemolysin did not act uniformly on the red blood cells from different animals. For example, the hemolysin was highly active against rat erythrocytes; moderately hemolytic for rabbit red blood cells; slightly hemolytic for sheep erythrocytes and inactive against horse red blood cells. Miwatani (31) and Miyamoto (34) later verified most of these reactions.

Crude hemolysin preparations exhibited a typical Arrhenius effect (32). The hemolysin was inactivated upon heating at 60°C for 10 minutes, but after 10 minutes at 90-100°C there was no inactivation. At this point it became obvious that the isolation and purification of the hemolytic substance was necessary.

A scheme to obtain hemolysin from V. parahaemolyticus was developed (41) using techniques established for the purification of toxin from V. cholerae. After growth in broth culture, the cells were removed by centrifugation and the active material precipitated from the supernatant

with ammonium sulfate. This was followed by dialysis and reprecipitation with acetic acid. This material was applied to an anion exchange column followed by gel filtration (31, 41).

Purification of crude hemolysin resulted in the isolation of both heat labile and heat stable fractions. One fraction of purified hemolysin (thermostable hemolysin) was not inactivated at either 60°C or 100°C for 10 minutes. However another factor, found with the hemolysin in crude preparations was inactivated by heating at 100°C (32, 39, 41, 42, 46). Thermostable hemolysin was found in Kanagawa positive but not negative strains of V. parahaemolyticus.

Antibody prepared against the thermostable hemolysin inhibited all hemolytic activity and the activity of Kanagawa positive cultures. The thermostable hemolysin was thus shown to be responsible for the beta-hemolysis of V. parahaemolyticus (42).

Sakurai (39) demonstrated a cation effect on the interaction of hemolysin and erythrocytes. The neutralizing effect that antiserum had on the hemolytic substance decreased in the presence of 25 mM calcium, magnesium or manganese ions but not with potassium. The inhibitory effect of antiserum also decreased with decreasing pH (minimum pH = 6.0) and was independent of temperature (39).

Temperature studies have shown that, in the hemolytic process, a lag time exists after the hemolysin becomes

bound to the erythrocytes. Because hemolysin absorbs to, but does not lyse erythrocytes at low temperatures, the act of absorption was distinguished from subsequent events leading to hemolysis (39). Free hemolysin was "neutralized" by antiserum, but it was not clear if antiserum effects hemolysin absorbed to the surface of red blood cells.

Other properties determined include molecular weight estimation, isoelectric point and amino acid analysis. The molecular weight was estimated to be 118,000 or 114,000, depending upon the technique used (41). The isoelectric point was 4.5 and the amino acid analysis revealed that there were approximately 43% acidic and 17% basic amino acids.

In order to study the toxicity of the hemolysin, several animal test systems were used. The most popular method to determine enteropathogenicity was by the use of the ligated rabbit ileal loop. It was generally accepted that a strain's hemolytic ability was related to its capacity to produce fluid accumulation and dilation in the ligated ileal loop. Results obtained by Johnson and Calia (23) and Brown et al. (10) question the reliability of all previous work done using the ileal loop technique.

Recently, new methods for hemolysin purification were published (34, 46, 47). Of these, Takeda et al. (47) presented one which was quite effective. Using this technique as a basis, Honda et al. (21) modified the protocol and

produced a better method to prepare purified toxin from V. parahaemolyticus.

After growth of V. parahaemolyticus in a broth medium, the cells were removed by centrifugation and the hemolysin was precipitated from the supernatant by ammonium sulfate (55% saturation). This preparation was then applied to a DEAE-cellulose column and positive fractions were reprecipitated with ammonium sulfate. The dialysate was applied to a hydroxyapatite column and further concentrated with a collodion bag. Finally, gel filtration with Sephadex G-200 was used followed by further concentration of the active substance (21).

This preparation was shown, by analytical ultracentrifugation and sodium dodecyl sulfate polyacrylamide gel electrophoresis, to contain a single protein. The toxic activity was determined using mice and the hemolytic assay used was developed by Miwatani et al. (32), Sakurai et al. (41) and Honda et al. (21). It was shown that the purified lethal toxin of V. parahaemolyticus was identical to the hemolysin (21). These results verified the postulated lethal toxin described by Ueyama et al. (51).

The properties of this purified toxin differed slightly from most of the characteristics stated in earlier reports. The toxin was pure protein, that is 500 µg did not contain detectable amounts of carbohydrate or phospholipid. The sedimentation coefficient was 6.9S and the molecular weight

was about 42,000. In review, Zen-Yoji's molecular weight estimation of 45,000 (56) and Miyamoto's (19) estimation of 44,500 correlated most closely with Honda et al.'s results. The isoelectric point was approximately pH 4.2 and the amino acid composition was as shown in Table 8 (21).

The exotoxin preparation was injected intraveneously into small animals and, unexpectedly, was found to severely effect the heart (19). When 0.1 µg of purified toxin was added to cultured heart cells, the beating stopped within 1 minute. Destruction of heart cells was caused by addition of 1.0 µg of toxin. Other cell lines affected include FL, HeLa, L and Elrlich tumor cells (40).

A mixture of gangliosides was found to inhibit the hemolytic activity of the toxin. In this investigation Takeda et al. (49) had subsequently found that the neuraminidase sensitive G_{T1} ganglioside was the most effective inactivator of the toxin's hemolytic and lethal activity.

It was then suggested (21, 40, 48, 49) that a ganglioside might be the membrane receptor site for the toxin of V. parahaemolyticus. Sakurai (40) proposed that the difference in sensitivities of various red blood cells, to the action of the toxin, might be explained by the differences in the amounts of gangliosides present on the erythrocyte surfaces. A ganglioside mixture extracted from horse erythrocytes, to which the exotoxin was not hemolytic, did not contain the neuraminidase sensitive gangliosides. However

these essential compounds were present in exotoxin sensitive erythrocytes such as human red blood cells (48).

Since the discovery that the crude toxin demonstrated a peculiar response to heating, there has been speculation as to the nature of V. parahaemolyticus toxin. It was thought that some factor, naturally occurring with the toxin but removed during purification, was responsible for this effect. Takeda et al. (45, 46, 47) found that a factor could be separated from the toxin by DEAE-cellulose column chromatography. This inactivating factor was found to be solely responsible for the thermally activated destruction of toxin at 60°C (45, 46). Evidence suggests that the loss of hemolytic activity was not due to an aggregation of toxin in the presence of inactivating factors. Instead, perhaps the factor has a proteolytic activity when heat stimulated at 60°C and thus destroys the otherwise thermostable toxin (47). After incubation at 80-100°C the toxin retains activity and the factor is destroyed. It was also shown that this inactivating factor was enhanced by sodium and magnesium ions, and was most active at pH = 8.0 (47).

Research has been done by Honda et al. (20) in an effort to ascertain why such a large amount (200-500 µg) of toxin was needed to give a positive ileal loop test. A factor having choleragen like properties was suspected and an isolation procedure for its purification was described.

The only properties reported about this proposed factor were that it was not hemolytic, absorbed strongly at OD_{280} and was eluted off a DEAE-cellulose column with .5M sodium chloride (20).

Currently there is an interest in the genetic origin of toxin production in V. parahaemolyticus. It was suggested by Baross (J. A. Baross, Ph.D. dissertation, University of Washington, Seattle, 1972) and later by Barker et al. (3) that the ability to produce hemolysin might be plasmid mediated. Initial results of research done by Guerry and Colwell (18) indicate that there was no close correlation between the ability to produce hemolysin and the presence of a plasmid(s).

The possibility that bacteriophage interaction among vibrio species was responsible for the Kanagawa phenotype was first implied by Baross (J. A. Baross, Ph.D. dissertation, University of Washington, Seattle, 1972). That is, because a high number of bacteriophages and a diverse vibrio group coexist in shellfish, it was suggested that most marine vibrios were associated with bacteriophage (7). This association might allow for transduction to occur among many marine vibrios.

The ability to digest agar has already been shown to be transmitted to V. parahaemolyticus by a phage isolated from a psychrophilic agar digesting vibrio (7, 8). In a similar manner bacteriophage might be an important and yet

unconsidered factor in explaining other characteristics of marine vibrios. The often transient ability of V. parahaemolyticus to produce a hemolytic exotoxin, that is correlated with human pathogenicity, might also be a bacteriophage mediated event (7).

MATERIALS AND METHODS

Preparation of Sample for Examination

Shellfish including soft shell clams (*Mya arenaria*), bay mussels (*Mytilus californianus*) and cockles (*Clinocardium nuttali*) were collected in the summer from Yaquina Bay, Oregon. Shucked Pacific oysters (*Crassostrea gigas*) were obtained commercially.

The shellfish samples were processed within 6 hours after collection, following procedures described by Hunt *et al.* (22). All instruments and materials used to examine the samples were sterile. The external surfaces of the shellfish were cleaned and the entire internal animal was removed into a tarred beaker.

A 1:2 (W/V) dilution was made by transferring 50 g of the weighed sample to a laboratory blender jar with 50 ml of 3% NaCl, pH 7.0. Also, 1:10 (W/V) dilutions were made by adding 450 ml of 3% NaCl to 50 g of shellfish sample. This was homogenized for 1 minute in a laboratory blender at approximately 8,000 r.p.m. Several sets of diluted samples were prepared so that all of the collected shellfish was used.

The commercially obtained shucked oysters were also weighed, diluted and blended as described.

Bacteriological Procedures

Prepared shellfish samples were enriched in Glucose Salts Teepol Broth (GSTB) followed by selective isolation on Thiosulfate-Citrate-Bile salts-Sucrose agar (TCBS). GSTB was used as a general enrichment broth for vibrios, it is specifically recommended for the enrichment of Vibrio parahaemolyticus (36).

Ten-fold serial dilutions (V/V) of the 1:10 sample were made in 3% NaCl, and 3-10 ml portions of this dilution were inoculated into 10 ml of double strength GSTB. Three 1 ml portions of 1:10, 1:100, 1:1,000 and 1:10,000 were inoculated into single strength GSTB. The tubes were incubated for 18 hours at 35°C. Duplicate tubes were prepared and incubated at 8°C for 72 hours.

Additionally, TCBS plates were directly inoculated with 0.1 ml of the 1:2 dilution and 0.1 ml of the 1:10 dilution. Duplicate plates were prepared and incubated at both the same high and low temperatures as the GSTB tubes.

All tubes showing turbidity were transferred to TCBS agar. One loopful of broth from turbid GSTB enrichments was transferred to duplicate TCBS agar plates. Incubation was at 35°C for 18 hours and 8°C for 72 hours.

Each plate was examined for typical blue green centered colonies, 2-3 mm in diameter, characteristic of V. parahaemolyticus. Also, slightly atypical lighter green

colonies were noted. Plates with confluent growth or colonies closer than 0.5 mm were discarded. Five green colonies, including both typical and atypical, were randomly picked from each plate. This procedure produced 235 vibrio isolates that were restreaked on TCBS agar to insure cultural purity.

One colony was picked from each new plate and transferred to a Lib-X agar slant (4). After confluent growth had developed, the cultures were stored for further testing. Isolates that had been consistently grown at 35°C were stored in duplicate at 8°C and 20°-22°C (room temperature). Cultures grown only at 8°C were held at 6°-7°C for storage.

Bacterial morphology and swarming were observed from fresh cultures grown in Lib-X agar or broth incubated at either 8°C, 20°-22°C or 35°C. Observations were made of gram stained preparations and by phase contrast microscopy. Colony morphology was noted on Lib-X agar, TCBS agar and Wagatsuma agar plates.

A well defined, clear, beta-hemolysis produced on a specially prepared medium (Wagatsuma agar) has been termed the Kanagawa phenomena by Japanese investigators (33). Directions for the preparation of Wagatsuma agar were those described by Leininger (26). (It was important to adjust the medium pH with something other than Tris, or

any other compound with a detergent action.) The adjustment of the final pH with 1N NaOH worked well. Also, the 2% (V/V) rabbit erythrocytes must be slowly warmed to 37°C before the addition to the medium base. A high quality commercially obtained (Prepared Media Laboratory, Tualatin, Oregon), sterile, defibrinated rabbit blood was used throughout this study.

A freshly prepared plate of Wagatsuma agar should appear as an opaque, solid red medium similar to a standard sheep blood agar plate. This medium should not appear transparent at any time (from lysed erythrocytes) or very dark (from the addition of too much crystal violet). Variations in medium ingredients or procedural technique upset this test. Additionally, the use of known positive and negative strains as controls was essential, especially at temperatures above 20°C.

The ability of each isolate to produce a beta-hemolysis on Wagatsuma agar was tested. Each isolate was inoculated onto Wagatsuma agar and tested for hemolysis in a humidified chamber at various temperatures. Isolates incubated at 37°C were read in less than 24 hours. Cultures incubated at 30°, 22°, 15° and 8°C were read at 24 hours, 45-75 hours, 3-5 days and 3-8 days, respectively. All reactions were evaluated well in advance of any spontaneous lysis in the medium.

The biochemical tests used in this study were those specifically outlined (36) for the identification of vibrios. All tests, media, and other materials were made according to standard procedures (9, 26, 36).

All media contained an additional 3% (W/V) NaCl. Except where otherwise stated, both the higher temperature of 35°C for 24 hours and lower 8°C temperature for 3 days were used for incubation. This was done in continuity with the temperatures used for enrichment and isolation of the cultures.

All 235 isolates were immediately subjected to tests to determine the oxidase reaction, motility, salt requirements and Triple Sugar Iron agar (TSI) reactions. Further biochemical tests were then done on selected isolates.

The presence of oxidase was determined by using .5% tetramethyl-p-phenylenediamine HCl (Eastman Kodak Co.). Saturated filter paper strips were rubbed with portions of colonies removed by wooden applicator sticks (9).

Motility was checked using Edwards and Ewing motility medium (BBL) with .05% added triphenyltetrazolium chloride (9).

A broth medium containing 1% trypticase (BBL) adjusted to pH 7.5 was used to test the ability of the isolates to grow in various amounts of NaCl. The concentrations of added NaCl were 0%, 6%, 8%, and 10% (W/V).

Isolates were inoculated into TSI medium and incubated at 8°C for 3-7 days, 20°-22°C for 2-5 days or 35°C for 18-24 hours. Results were recorded at the lower temperatures after visible growth appeared.

The temperature growth range was determined for all Kanagawa positive strains isolated using Lib-X broth. A 1% (V/V) inoculum of an overnight broth culture, at approximately 10^7 cells/ml, was used. A temperature gradient incubator (Scientific Instruments, Inc., model TN-3) was used and all tubes were temperature equilibrated before being inoculated. To avoid cell death by thermal shock, the cultures used for inoculation were grown at 8°C for temperature growth tests ranging from 0°-20°C, and at 20°-22°C for those tests at 20°-50°C.

Purple Broth Base (Difco) was used to check for the fermentation of various carbohydrates. The carbohydrates were prepared separately in 10% solutions, sterilized by autoclaving, and added to 10 ml portions of broth. Mannitol, maltose and trehalose were added in a final concentration of 0.5%. Sucrose and cellobiose were added in a 1% final concentration. A sterile mineral oil overlay was added to the sugar broths.

Hugh Leifson medium (BBL) was used for the determination of glucose oxidation or fermentation (9).

Decarboxylation of lysine and ornithine and the dihydrolation of arginine was tested using the medium of

Moeller (Difco). Inoculation was from a young culture and a 4-5 mm layer of sterile mineral oil was added. A control tube with no added amino acid was also inoculated for each isolate tested (9).

Gelatin liquification was demonstrated by preparing a stab culture in nutrient gelatin (Difco). The tubes were held for at least 5 days at either a high or low incubation temperature (9).

The Voges-Proskauer test was done by using MR-VP medium (Difco). After incubation the presence of acetyl-methyl carbinol was checked by the modified O'Meara method using potassium hydroxide and creatine (9).

Indole production was determined by the addition of Kovac's reagent to cultures grown in 1% tryptone broth (Difco) (9).

Preparation and Testing of Crude Hemolysin

Selected isolates were grown on a shaker in Lib-X broth for 15-17 hours at various temperatures. Cells were removed by centrifugation at 4080 x G for 30 minutes using a Sorvall refrigerated (5°C) centrifuge. The pellet was discarded and the supernatant was centrifuged twice more until a clear preparation was obtained. The cleared supernatant was used as a crude hemolysin.

The hemolytic assay was a modification of the procedure used to detect the hemolytic toxin of *V.*

parahaemolyticus (21). A two-fold serial dilution of cleared supernatant was made using 1 ml of the crude hemolysin, 1 ml diluent (10 mM Tris, 5 mM $CaCl_2$, 0.9% NaCl pH 7.2) and 2 ml of a 1% suspension of rabbit red blood cells.

To prepare a 1% suspension of red blood cells, the cells were first washed three times in physiological saline (pH 7.2) and reconstituted to the original volume of blood. Dilutions of the blood were then made in physiological saline.

Incubation was for 1 hour at 30°C followed by centrifugation at 5000 rpm for 5 minutes in a desk top clinical centrifuge. Hemolytic activity was determined by measuring the levels of hemoglobin released from lysed red blood cells using a spectrophotometer at 540 nm.

The temperature range of hemolytic activity was tested on selected organisms grown at 8°C for 60-72 hours on a shaker in Lib-X broth. A cleared supernatant was prepared. A hemolytic assay was performed using serial 2-fold dilutions of cleared supernatant. Incubation was at 37°, 30°, 22°, 15° and 8°C for 3 hours. Red blood cells without hemolytic supernatant were used as the control at each temperature.

The effect of proteolytic enzymes on the crude hemolysin was tested using pronase (Grade B, Calbiochem) and protease (Type VI, Sigma). Stock solutions of each enzyme were prepared in 0.01 M phosphate buffer (Na_2HPO_4-KH_2PO_4 pH

7.5) at a 1 mg/ml concentration. Enzymes were prepared just prior to use and predigested at 35°C for 10-15 minutes.

Stock enzyme preparations were added to portions of crude hemolysin to yield final concentrations of 0.01, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. Controls to test the effect of these concentrations of proteolytic enzymes on a 1% solution of rabbit erythrocytes were also done.

Incubation was for 3 hours at 20°-22°C after which each solution was assayed for hemolytic activity.

Isolation and Partial Purification of Hemolysin

Initially, the scheme developed by Honda et al. (21) for purifying hemolytic toxin of V. parahaemolyticus was tried. However, due to differences in the properties of the hemolysins, the published protocol was continuously modified each time the procedure was attempted.

Throughout the purification and characterization of the hemolysin, the glassware that was used was specially cleaned (12).

A selected isolate was grown on a shaker at 20°-22°C for 15 hours in Lib-X broth. A 1% inoculum of an actively growing culture, approximately 10^7 cells/ml, was used. The broth culture was centrifuged three consecutive times for 30 minutes at 5860 x G to insure a clear supernatant. The specific activity of the preparation was then determined.

The specific activity of a hemolysin is defined as the total number of hemolytic units present in the preparation divided by the total mg of protein present. One hemolytic unit is the amount of hemolysin necessary to produce an absorbance value of 0.5 at 540 nm.

The hemolytic assay was as previously described with the exception that the incubation period was 30 minutes at 30°C. As purification progressed, smaller volumes of hemolysin were used, but the total volume of the reaction mixture was kept constant by the addition of 0.02 M phosphate buffer (KH_2PO_4-Na_2HPO_4, pH 7.0).

Protein was measured by the technique of Lowry (17, 28). Bovine serum albumin (Sigma) was used as the standard protein at concentrations of 10 µg/ml to 100 µg/ml in 10 µg increments. Samples were measured at 670 nm on a Spectronic 20 (Bausch and Lomb).

Initially various saturation levels of ammonium sulfate were tried to differentially precipitate the hemolysin. This was done in 5% increments from 0%-55% saturation at 0-4°C. The hemolytic activity of each cut was then assayed. However, the hemolysin yield was low and results were difficult to reproduce, so a 55% saturation level was ultimately selected. Solid ammonium sulfate (Analytical Reagent, Mallinckrodt) was added, approximately 10% per 30 minutes at 0-4°C in an ice bath. After the final addition of salt (32.6 grams/100 ml cleared supernatant, 52) the

solution was allowed to mix for 24 hours at the same temperature. The precipitate was collected by centrifugation at 4080 x G for 20 minutes using a Sorvall refrigerated (5°C) centrifuge.

Dialysis tubing was prepared and handled specifically for protein purification (30). Dialysis was against 0.02 M phosphate buffer for 15 hours at 8°C with 3-4 changes of (2500 ml each) buffer.

The hemolytic dialysate was tested for specific activity and then applied to a 2.5 cm x 40 cm DEAE-cellulose (Whatman DE-52) column. The column was prepared according to manufacturer's (Whatman Inc., 9 Bridewell Pl., Clifton, NJ 07014) directions. The general operational procedures were as previously described (37, 54). This column was equilibrated with the same phosphate buffer. Completion of equilibration was verified by measurement of pH and conductivity. All column work was done at 8°C.

After the hemolysin was applied to the column, 1000 ml of phosphate buffer was pumped through. No hemolytic activity was found in this waste effluent. Several different gradients with a narrow range of salt molarity were tried, but none eluted the toxin efficiently. One thousand ml of a linear gradient of 0-1.0 M NaCl in phosphate buffer was used to remove the protein in 10 ml fractions collected at a flow rate of 80 ml/hour. A hemolytic assay and a protein determination were done on all fractions.

The hemolytic fractions were pooled, tested for specific activity and precipitated with 70% solid ammonium sulfate (43.6 grams/100 ml solution) as previously described. Lower saturations of salt left hemolytic activity in the solution and higher concentrations precipitated non-hemolytic compounds.

Dialysis was as described except for the addition of 0.25 M NaCl to the phosphate buffer. It was discovered that the addition of 0.25 M NaCl to the protein preparation from this point on minimized the loss of specific activity.

The Sephadex column was prepared and used according to the manufacturer's directions (Pharmacia Fine Chemicals, Inc., 800 Centennial Avenue, Piscataway, NJ 08854).

The hemolysin preparation was applied to a 2.5 cm x 40 cm Sephadex G-100 column. The volume of the preparation was less than one-third the void volume of the Sephadex column. The column had been stabilized with phosphate buffer containing 0.25 M NaCl and the packing efficiency checked with Dextran Blue. Elution was carried out using 500 ml of phosphate buffer with 0.25 M NaCl and 5 ml fractions were collected at a flow rate of 30 ml/hour under 100 mm operating pressure. Hemolytic activity and protein determinations were done on each fraction. The hemolytic fractions were then pooled and tested for specific activity and protein content.

See Figure 1 for the flow diagram of the purification scheme.

Characterization of Partially Purified Hemolysin

One hemolytic unit of partially purified hemolysin was added to 1 ml of diluent and brought to a final volume of 2 ml with 0.02 M phosphate buffer containing 0.25 M NaCl. Phosphate buffer (KH_2PO_4-Na_2HPO_4 pH 7.0) with 0.25 M NaCl (SP buffer) was used throughout the remaining characterization tests unless otherwise noted. Two ml of 1% rabbit blood were added and the mixture was allowed to incubate for 1 hour at 37°, 30°, 20°-22°, 15° or 8°C. Red blood cell controls were prepared and incubated at each temperature. The hemolytic assay was performed as described.

SP buffer was temperature equilibrated (Temp-Blok Module Heater, Scientific Products, Model 2090). One hemolytic unit was added to the heated buffer and allowed to incubate for 12 minutes at the specified temperature. After incubation 1 ml of cold diluent was added to the mixture to bring the total volume to 2 ml. A 2 ml portion of a 1% suspension of rabbit erythrocytes was added. Red blood cell controls were prepared and the hemolytic assay performed as usual (45, 47).

Stock cultures of pronase and protease were prepared as previously described. Twenty µg of hemolysin was mixed with either 0.5 ml of a 1 mg/ml solution of pronase or

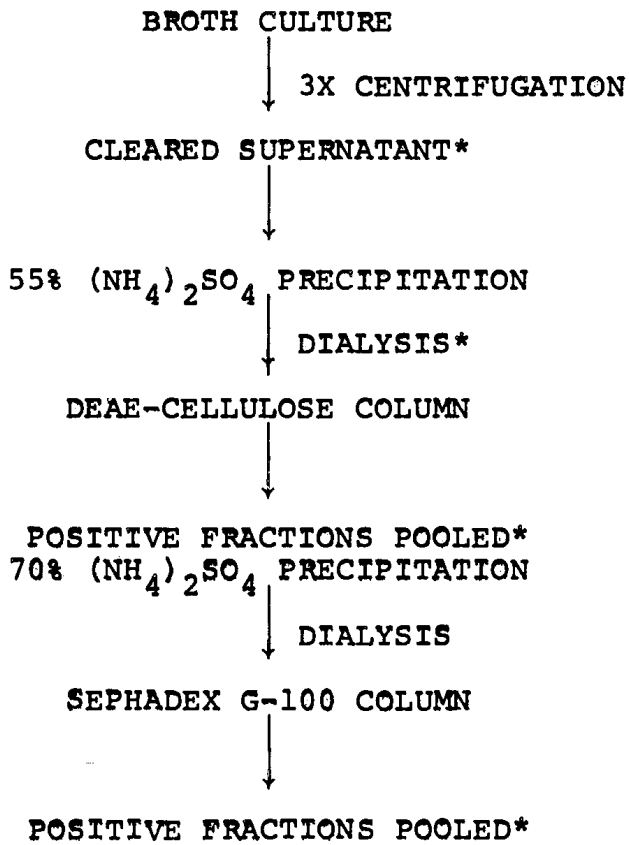

Figure 1. Flow diagram of the partial purification of hemolysin.

*The hemolytic unit and protein content were assayed to determine the specific activity

protease. Incubation was at 20°-22°C for 4 hours. Twenty µg of hemolysin (q.s.to 1 ml with SP buffer) were also incubated under the same conditions as a control. All tubes were then subjected to a standard hemolytic assay.

The carbohydrate content of 350 µg of partially purified hemolysin was measured by anthrone reagent (35). Glucose was used as a standard in concentrations ranging from 10-200 µg in 10 µg increments. Negative controls were made by using SP buffer. Color development was measured at 620 nm on a Spectronic 20 (Bausch and Lomb).

Fifty µg of hemolysin was mixed with increasing amounts of ganglioside (Type II, Sigma). Incubation was for 1 hour at 20°-22°C followed by a hemolytic assay. Effects of the various concentrations of gangliosides on rabbit red blood cells were also tested (21).

Sterile defibrinated horse blood was processed and prepared in a 1% solution in the same manner as was rabbit blood. Also the same hemolytic assay, substituting horse for rabbit erythrocytes was used. The degree of lysis caused by 1 hemolytic unit was measured.

Approximately 10-15 ml of Diffusion Agar was poured into each glass or plastic petri dish (11). Wells 2-3 mm in diameter were made with a sterile No. 1 cork borer and placement was 7-10 mm apart.

The control antigen, purified toxin (vibriolysin) from V. parahaemolyticus WP-1, its homologous antibody

(antivibriolysin) and the partially purified hemolysin were diluted in 0.02 M phosphate buffer-physiological saline containing 0.37% Brain Heart Infusion (Difco) and 0.01% thimerasol, pH 7.4 (personal communication, Dr. T. Morita; 13). This was used for all dilutions in preparation for the Ouchterlony precipitation test.

According to directions, the vibriolysin (1 mg/ml) was diluted 1:20 and the antibody diluted 1:32. Additional antibody dilutions of 1:5, 1:10, 1:15 and 1:20 were also prepared.

Portions of the partially purified hemolysin were either concentrated further or diluted to test the antigenic reactivity with the antivibriolysin. The hemolysin, originally in SP buffer, was diluted 1:2, 1:3, 1:4, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50 and 1:100. In addition, the undiluted hemolysin, 44 µg protein/ml was tried. The same protocol was also used without the added NaCl in the original phosphate buffer.

To concentrate the hemolysin several portions were dialyzed against a lowered molarity of phosphate buffer. After lyophilization and rehydration one portion contained hemolysin in 0.02 M phosphate buffer with 0.25 M NaCl, and the other portion had no additional NaCl. The hemolysin was concentrated approximately 45-fold to 2 µg/µl.

Increased concentrations of hemolysin, 2 µg/µl, 1 µg/µl and 0.5 µg/µl were tested for reactivity with

vibriolysin. The amount of protein added to the wells was 40 µg, 20 µg, and 10 µg, respectively.

The wells, in each case, were filled completely and incubation was at 20°-22°C or 8°C in a humidified chamber for up to 2 weeks.

Hemolysin preparation for analysis on a sodium dodecyl sulfate (SDS) polyacrylamide gel included dialysis, lyophilization and resuspension in SDS sample buffer.

Sodium Dodecyl Sulfate (SDS) Sample Buffer 2x*

Tris (Sigma)	0.756 g
Sodium Dodecyl Sulfate	2.0 g
Glycerol	10 ml
B-mercaptoethanol	5 ml
Bromphenol blue	0.001 g
Distilled water	35 ml
pH	6.8

*Georgia Riedel, personal communication

NOTE: Store in Nalgene containers.

A portion of hemolysin (44 µg protein/ml) containing 500 µg of protein was dialyzed against phosphate buffer which was 23 times less concentrated than the usual 0.02 M phosphate buffer used. Dialysis was as previously described and the material was lyophilized after quick freezing (Refrigeration for Science Freeze Dryer).

One portion containing 125 µg of protein was rehydrated with 62.5 µℓ distilled water and 62.5 µℓ of 2x SDS sample buffer. This 1 µg/µℓ protein solution was a 23 fold concentration of both protein and phosphate salts, without the additional 0.25 M NaCl.

Also, 125 µg of lyophilized hemolysin was rehydrated in 62.5 µℓ each of 0.25 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.0) and 2x SDS sample buffer. The final concentration was 1 µg/µℓ protein in SDS buffer.

Standards were prepared in 0.02 M and 0.25 M phosphate buffer to a final concentration of 1 µg/µℓ in SDS sample buffer. The standards used were bovine serum albumin (BSA) (MW 68,000; 5x recrystalized, Sigma), Aldolase (MW = 40,000; Grade 1, Sigma), α-chymotrypsinogen A (MW = 25,700; 6x recrystalized Type II, Sigma) and cytochrome c (MW = 12,400; Type VI, Sigma)(43).

A 1 mm thick 15% polyacrylamide slab gel was prepared with a 6% polyacrylamide stacking gel. The samples were allowed to run on the gel for 24 hours at 95 volts at 22°C in running buffer. The tracking dye was electrophoresed to approximately 1 inch from the bottom of the gel.

Running Buffer*

Tris-HCl	6.05	g
Sodium dodecyl sulfate	1	g
Glycine	3.8	g
Distilled water	1000	ml

*Georgia Reidel, personal communication.

The gel was stained by immersion in a Coomassie Blue G-250 solution, covered and kept gently shaking for 8-12 hours at 22°C.

Coomassie Blue G-250 Stain*

Methanol	450 ml
Acetic acid	90 ml
Coomassie Blue G-250	25 g
Distilled water	460 ml

*Georgia Reidel, personal communication.

Destaining required several changes of destain solution over a 24-36 hour period. The gel, immersed in the destain solution was covered and kept gently shaking. The destain was a solution of methanol, acetic acid and water (53).

A standard curve was prepared by plotting electrophoretic mobilities for protein markers against the log of their molecular weight. The molecular weight of each prominent band was extrapolated from this curve.

The molecular weight was approximated using Sephadex G-100 following the manufacturer's directions. A Sephadex G-100 column (2.5 cm x 50 cm) was equilibrated, stabilized and operated as previously discussed. However, the length of the column was greater, thus the void volume was larger.

Standard protein markers were prepared in SP buffer at a final concentration of 5 mg/ml. The markers and

hemolysin were passed through the column separately and collected in 5 ml fractions. The markers were the same as those used for SDS polyacrylamide gel electrophoresis with the addition of ovalbumin (MW = 45,000; Grade III, Sigma).

The standards were detected in the fractions by absorption at 280 nm on a Beckman DB spectrophotometer. The hemolysin was identified based on activity using the hemolytic assay.

The peak elution volumes of the calibrating proteins were plotted against the logarithm of their molecular weight. The approximate molecular weight of the hemolysin was extrapolated from this standard curve (25).

A volume of hemolysin containing 100 µg of protein was used for amino acid analysis. The sample was dialyzed against distilled water, lyophilized, and hydrolyzed with 6.1 N HCl for 22 hours at 110°C in an evacuated, sealed tube. The sample was then evaporated and the diluting buffer (pH 2.2) was added. The sample was analyzed on a modified Beckman 120 B Amino Acid Analyzer (44).

RESULTS

Bacteriological Procedures

The tubes of GSTB inoculated and held at 35°C for 18 hours showed heavy growth from inocula representing 1 g and 0.1 g portions of shellfish. The inoculated tubes of GSTB held at 8°C for 72 hours also showed the same growth pattern.

The source and characteristics of the organisms isolated for this study are summarized in Table 1.

Some Properties of the Crude Hemolysin and Isolate Selection

The Kanagawa positive isolates that gave the strongest beta hemolysis (Table 2) were further examined (Table 3).

Isolate 123-S1 was selected for further study for several reasons. It produced a large amount of hemolysin that exhibited activity over a wide temperature range (Tables 2 and 3). The consistently rapid growth and reliable hemolysin production were also important. The biochemical characteristics of this isolate are presented in Tables 1 and 4. The biochemical profile of isolate 123-S1 is compared with marine vibrios in Table 4.

The temperature growth range was also determined. The minimum, maximum and optimum growth temperatures for 123-S1 were 0°C, 34°C, and 20-30°C, respectively. The minimum, maximum and optimum growth temperatures determined for *V.*

Table 1. Characteristics of the marine *Vibrio* isolates and *V. parahaemolyticus*.

	V. parahaemolyticus Kanagawa +	Vibrio Isolates (210)[1] Kanagawa -	Vibrio Isolates (24)[1] Kanagawa +	123-S1 Kanagawa +
Source	Japan	Clams, mussels, cockles, oysters	Clams, mussels, cockles	Clams
Tests[2]				
TCBS	Green	Green	Lt. green, Green	Lt. green
Gram reaction	-	-	-	-
Morphology	----------Curved/straight rods----------			
	0.5 µm by 1.5-3.0 µm	---------- 0.5 µm by 0.5-2.0 µm----------		
Motility	+	+	+	+
TSI	K/A--[3]	A/A--, K/A--	K/A--, A/A--	A or K/A--
Hugh-Leifson O/F Media	F+G-[4]	F+G-	F+G-	F+G-
Oxidase	+	+	+	+
Lysine Decarboxylase	+	+, -[5]	-	-
Arginine Dihydrolase	-	-,+	+	+
Ornithine Decarboxylase	+	+	-	-
Gelatin	+	+,-	+	+
Halophilism (NaCl) 6%, 8%	+	+	+	+
0%,10%	-	-,+	-	-
Growth at 42°C	+	+,-	-,+	-
Voges-Proskauer	-	-,+	-	-
Indole	+	+	+	+
Cellobiose	-	-,+	+,-	+(slow)
Sucrose	-	-,+	+,-	+
Maltose	+	+	+	+
Mannitol	+	+	+	+
Trehalose	+	+	+	+

[1] Number of isolates

[2] Morris *et al.* (36)

[3] K/A--: Alkaline slant/Acid butt gas- H_2S-

[4] F = Fermentative; G = gas

[5] Some strains positive and some negative

Table 2. Effect of temperature on the activity of Kanagawa positive marine vibrios on Wagatsuma agar.[1]

Organism	35°C	30°C	20-22°C	15°C	8°C
V. parahaemolyticus					
M5242	++	G[2]	G	NG	NG
SAK8	+++	G	G	NG	NG
K4	++	G	G	NG	NG
Vibrio spp. isolates					
101-0M	±[3]	±	±	NG	NG
101-0L	±	±	±	NG	NG
101-T	+	±	±	NG	NG
1 - 0	NG	NG	G	+	G
1 - C			+	G	G
3 - C			++	G	G
3 - SL			+	+	G
3 - SS			++	+	±
3 - LL			+	G	G
3 - LS			++	G	G
6 - L			G	+	G
81 - SS			+++	G	G
84 - C			++	G	G
87 - L		+++	++++	++++	++++
87 - M		+++	++++	++++	++
98 - L		++	++	G	++
112		++	++	G	+
113 - C		++	++	±	G
113 - 0		±	±	±	+
121 - C		+++	+++	++++	++++
121 - 0		++	++	++++	++
123 - S1		+++	+++	++++	+++
123 - S2		+++	+++	++++	+++
123 - L		+	+	++++	++

[1] See Materials and Methods for incubation conditions.

[2] G = growth only; NG = no growth.

[3] Beta-hemolysis demonstrated as a definite zone around an area of growth. All other reactions were considered negative.

- ± Equivocal hemolysis
- + Faint but definite clearing around colony edge
- ++ Small beta-hemolytic zone, 1-2 mm
- +++ Obvious beta-hemolytic zone, 2-3 mm
- ++++ Large beta-hemolytic zone, >5 mm

Table 3. Hemolytic activity of cleared supernatant at various temperatures.

Temperature	Selected Kanagawa positive isolates				
	87-L	81-SS	121-C	123-S1	123-L
35°C	0.44[1/]	0.42	0.44	0.46	0.50
30°C	0.48	0.41	0.42	0.47	0.51
20-22°C	0.42	0.42	0.37	0.48	0.50
15°C	0.23	0.12	0.10	0.48	0.34
8°C	0	0	0	0.44	0.06

[1/] Absorbance measured at 540 nm.

Table 4. Scheme for differentiating related marine vibrios.[1/]

Test	V. parahaemolyticus	V. alginolyticus	V. anguillarum	Undefined vibrios	123-S1
TCBS (green colony)	+	-	-,+[2/]	+,-	±
Growth at 42°C	+	+	-	-,+	-
Growth with					
0% NaCl	-	-	-,+	-,+	-
8% NaCl	+	+	-,+	-	+
10% NaCl	-	+	-	-	-
Lysine decarboxylase	+	+	-	+,-	-
Sucrose	-	+	+,-	-,+	+
Voges-Proskauer	-	+	-	-	-
Swarming on TSA, 3% NaCl, at 35°	-	+	-	-	-[3/]

[1/] Morris et al. (36).

[2/] Some strains -, and some +.

[3/] Test performed at room temperature (20°-22°C) and 30°C.

parahaemolyticus, however, were 9°C, 47°C and 30-35°C respectively.

The hemolytic action of the cleared supernatant was destroyed by preincubation with pronase and protease as seen in Table 5. The addition of greater than or equal to 0.2 mg/ml final concentration of enzyme inhibited hemolysis. The red blood cells were not lysed by pronase or protease at the concentrations that were used.

Partial Purification of Hemolysin

The protein purification scheme shown in Fig. 1 was the final result of a number of attempts to harvest the hemolysin from strain 123-51. In addition, the entire protocol was repeated four times to generate sufficient data on the hemolysin's characteristics.

On the DEAE-cellulose column (Fig. 2) the hemolysin was eluted in one broad peak with approximately 0.25-0.35 M sodium chloride. The positive fractions were pooled and the hemolysin reprecipitated with a 70% saturation of ammonium sulfate.

Dialysis of the 70% precipitate was followed by gel filtration with Sephadex G-100 (Fig. 3).

Table 6 compares the activity and protein content during the different phases of hemolysin purification.

The positive fractions were pooled and tested to determine some physical and chemical properties of the hemolytic preparation.

Table 5. Inactivation of crude hemolysin by proteolytic enzymes.

Final enzyme concentration[1] (mg/ml)	A_{540}
None	0.48
0.01	0.40
0.1	0.14
0.2	0.02
0.3	0
0.4	0
0.5	0

[1] Pronase or protease.

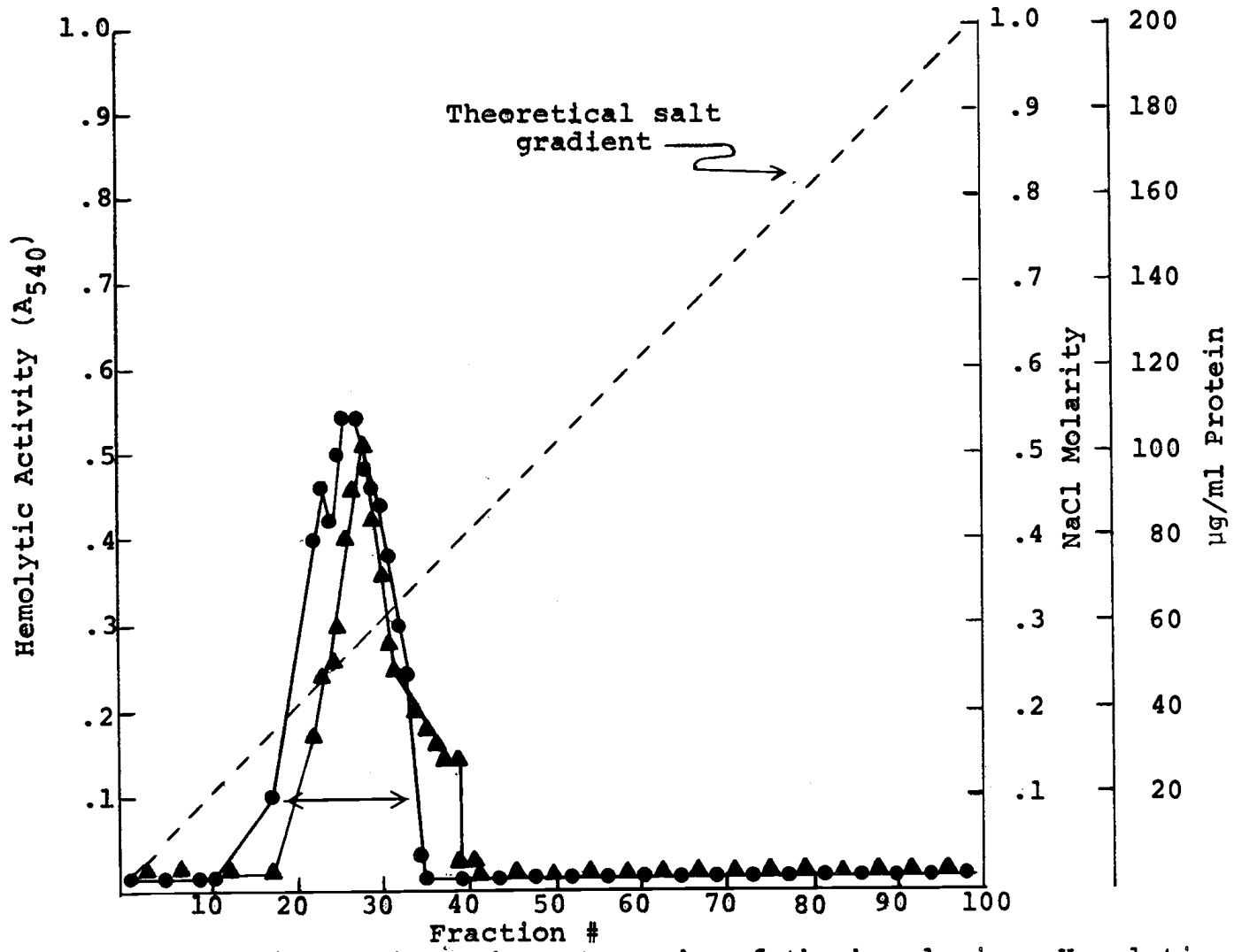

Figure 2. DEAE-cellulose column chromatography of the hemolysin. Hemolytic activity (●) and protein concentration (▲) were determined and the positive fractions were pooled (⟷).

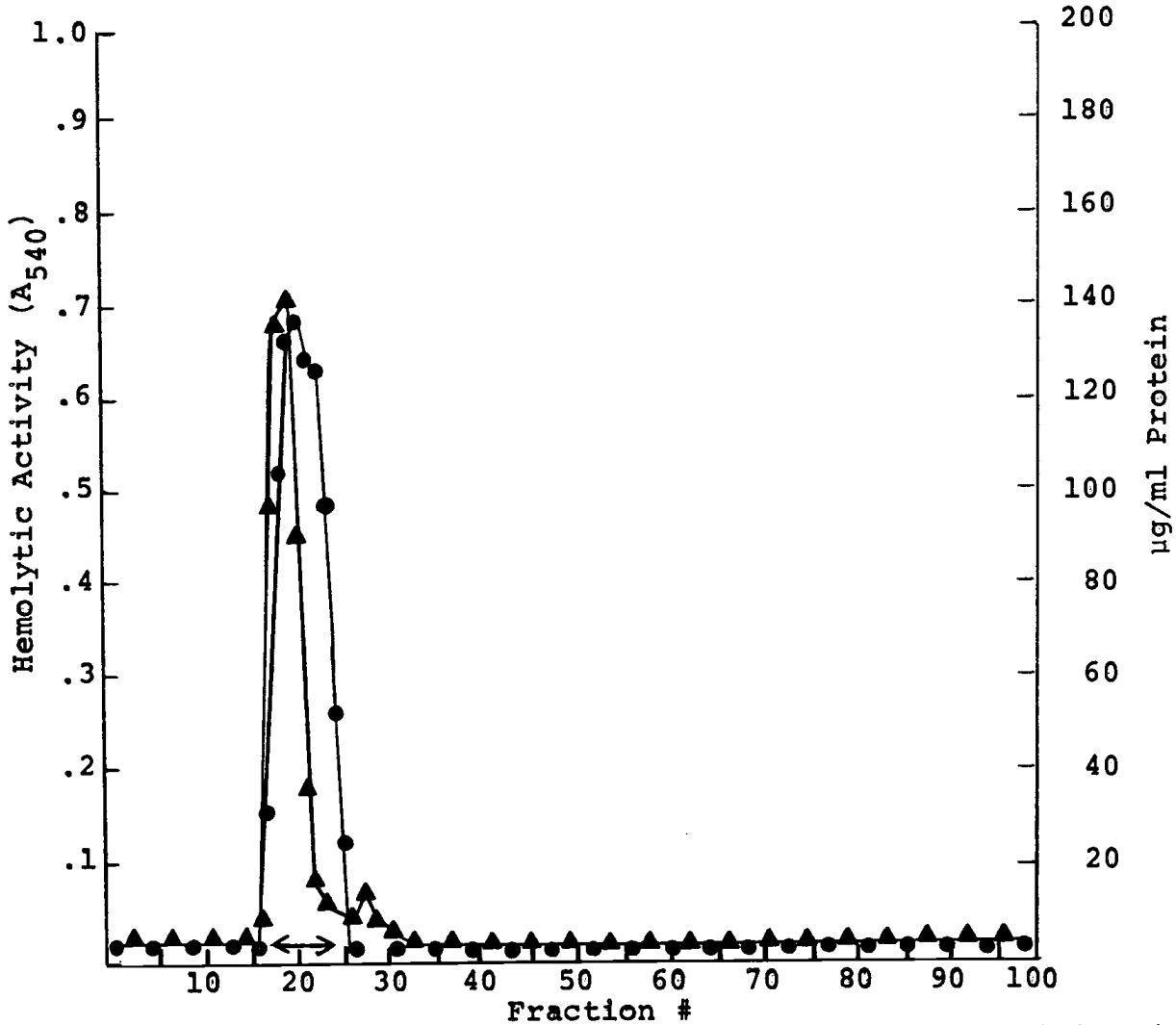

Figure 3. Sephadex gel filtration of the hemolysin. Hemolytic activity (●) and protein concentration (▲) were determined and the positive fractions were pooled (⟷).

Table 6. Partial purification of hemolysin from marine vibrio isolate 123-S1.

Sample	Volume (ml)	Total protein (mg)	Total activity (# hu)	Specific activity (# hu/mg)	Relative activity
Cleared supernatant	15,000	12,510	83,333	6.7	(1)
55% dialysate	255	47.2	4,250	90	13.5
DEAE-cellulose eluate	162	8.1	900	111.1	16.7
Sephadex G-100 eluate	42	1.9	191	102.6	15.4

Properties of Partially Purified Hemolysin

The temperature range of activity for the hemolysin was determined. There was an increase in hemolytic activity as the incubation temperature increased (Fig. 4). The rabbit red blood cells incubated as a control without hemolysin were stable at temperatures to 37°C. However, results from higher incubation temperatures were difficult to interpret due to erythrocyte thermolability.

The hemolysin preparation was heat sensitive, it became inactive after 12 minutes at 30°C or higher (Fig. 5).

Twenty µg of the hemolysin was inactivated by 0.5 mg/ml pronase or protease. A 1% solution of rabbit erythrocytes was not lysed by the concentrations of pronase or protease that were used.

The amount of carbohydrate in the partially purified preparation of hemolysin was 49 µg/ml.

The effect of a ganglioside mixture on the vibrio hemolysin is shown in Table 7. The ganglioside mixture does appear to have some effect on the hemolysin's activity. Maximum reduction in hemolysis was seen after preincubation with 1.0 mg ganglioside. Higher concentrations of ganglioside did not fully dissolve and therefore could not be tested.

The hemolysin showed substantially lowered activity to horse erythrocytes. One hemolytic unit, that produced an absorbance of 0.50 at 540 nm with rabbit erythrocytes,

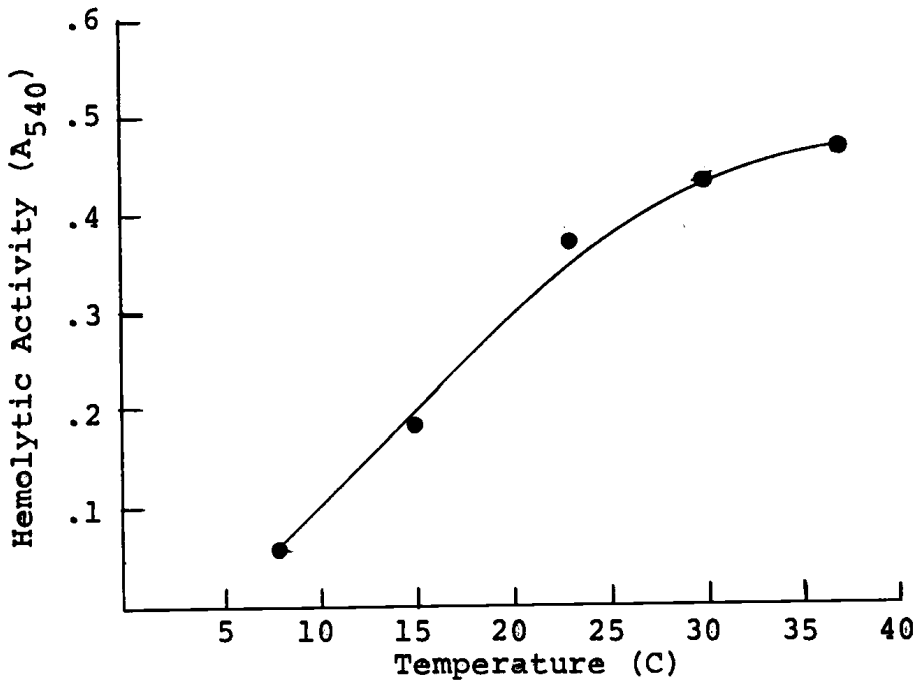

Figure 4. Temperature activity range of the hemolysin. The activity of the hemolysin was determined at increasing incubation temperatures.

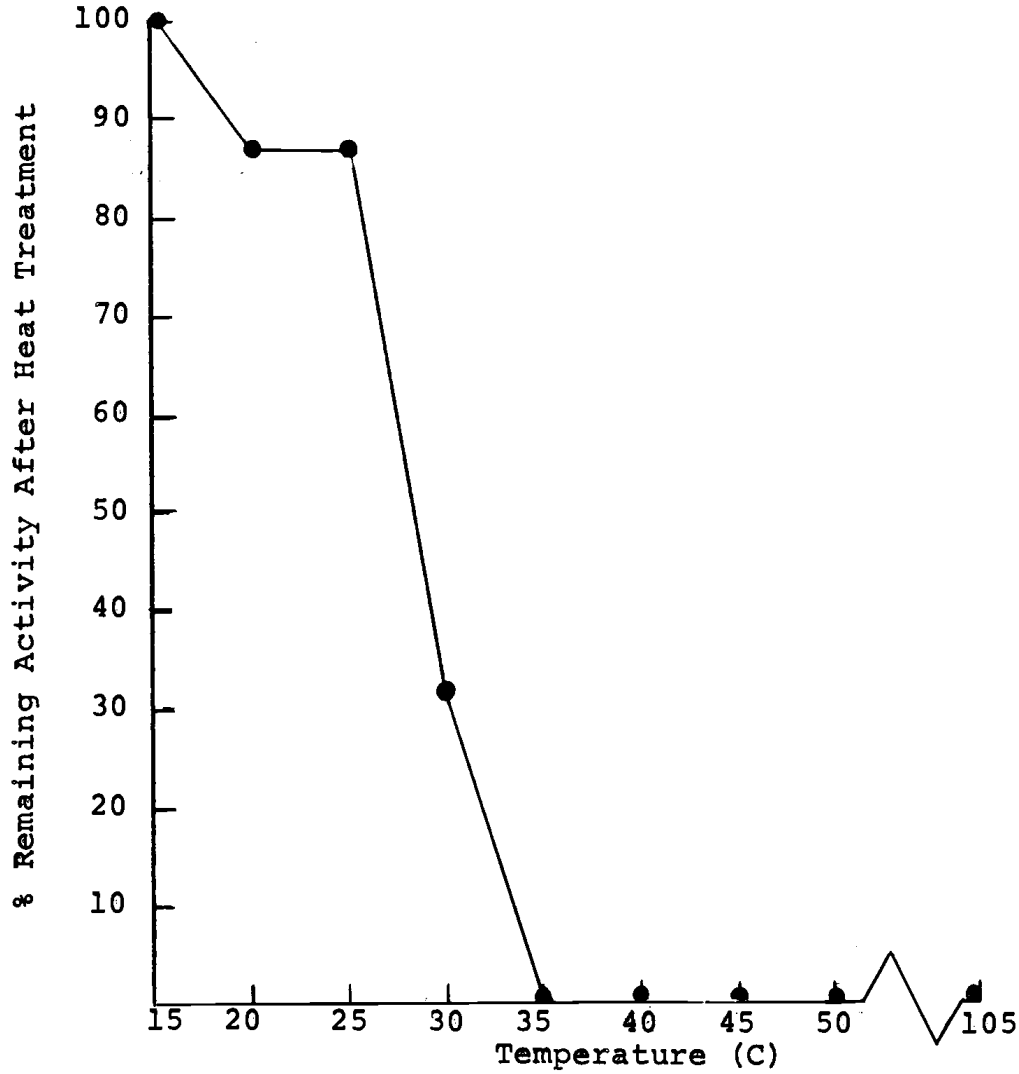

Figure 5. Thermal stability of the hemolysin. The hemolysin was first heat treated at increasing temperatures and then checked for remaining activity by the hemolytic assay.

Table 7. The effect of gangliosides on hemolytic activity.

mg ganglioside added	A_{540}	Percent remaining activity
None	0.58	100
0.1	0.53	91
0.2	0.46	79
0.3	0.45	78
0.5	0.43	74
1.0	0.38	66

produced an absorbance of only 0.25 when incubated with horse red blood cells.

Figures 6 and 7 show the results of the Ouchterlony gel diffusion tests. The optimal concentrations of vibriolysin and antivibriolysin necessary to form a precipitin line equidistant between both wells is illustrated in Fig. 6. The antigen diluted 1:20 and the antibody diluted 1:5 gave a sharp, clear line of precipitation within 24 hours at room temperature. The hemolysin preparation, variously diluted or concentrated, did not precipitate with the antivibriolysin (Fig. 7).

Electrophoresis on a SDS polyacrylamide gel revealed (Fig. 8) three major bands with approximate molecular weights of 55,000, 48,000, and 46,000. There were also lighter molecular weight constituents in the preparation that caused the smear seen near the midsection of the gel. Material heavier than the three prominent bands also appeared at the top of the gel. Because the gel displayed more than one band, the information gathered from this experiment was limited.

The characteristic banding patterns of the protein standards were not disturbed by the addition of 0.25 M phosphate buffer.

Also, it was observed that the hemolysin suspended in 0.25 M phosphate buffer maintained a reproducible banding pattern. However, hemolysin prepared in 0.02 M phosphate

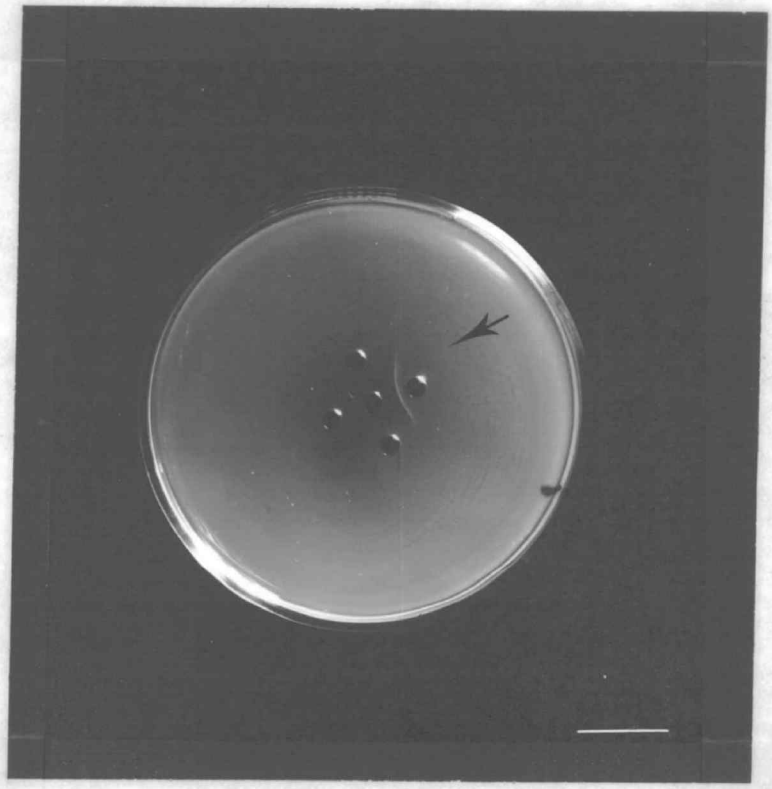

Figure 6. Ouchterlony gel diffusion control plate. The center well contains a 1:20 dilution of vibriolysin. The outer wells contain (clockwise from the arrow) antivibriolysin diluted 1:5, 1:10, 1:15 and 1:20.

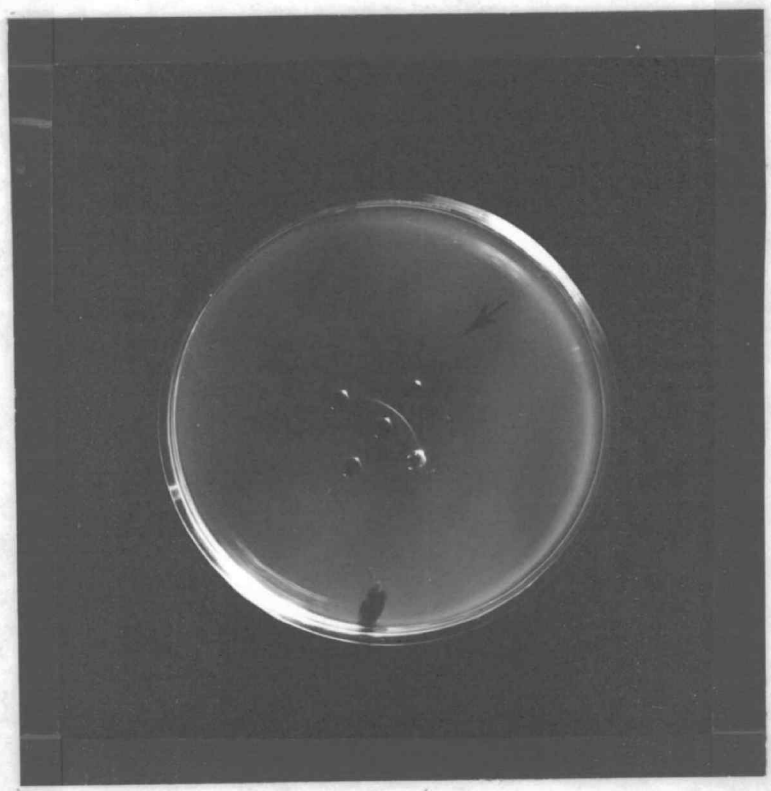

Figure 7. Ouchterlony gel diffusion of the hemolysin. The center well contains a 1:5 dilution of anti-vibriolysin. The outside wells contain (clockwise from the arrow) a 1:20 dilution of vibriolysin, 2 µg/ml hemolysin, 1 µg/ml hemolysin and 0.5 µg/ml hemolysin.

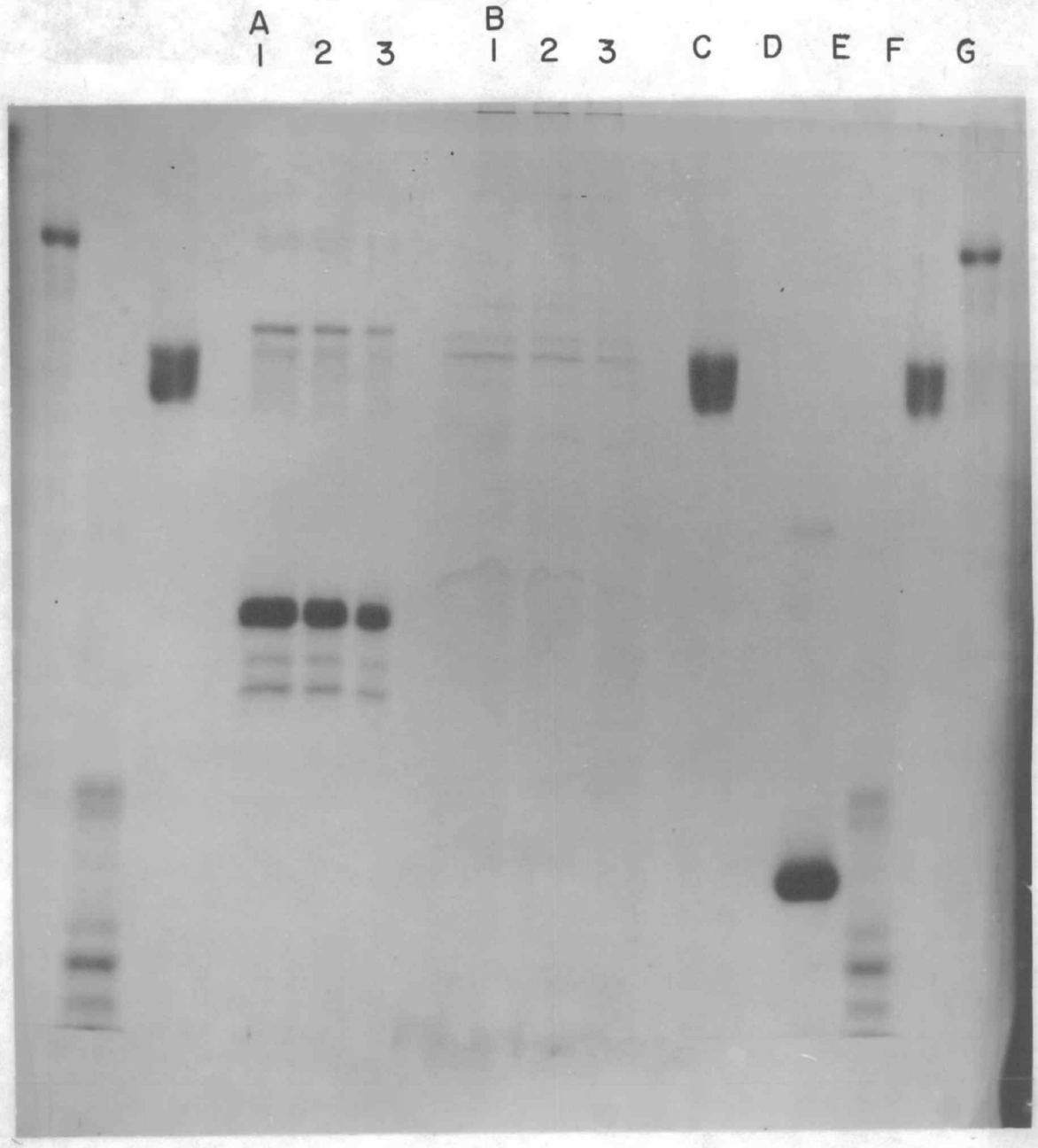

Figure 8. SDS polyacrylamide gel electrophoresis of the hemolysin. The wells contain: A 1-3 = 30 µl, 20 µl and 10 µl vibriolysin; B 1-3 = 30 µl, 20 µl and 10 µl of hemolysin; C = 7 µl aldolase; D = 7 µl cytochrome c; E = 7 µl α-chymotypsinogen A; and G = 5 µl BSA.

buffer gave a very erratic pattern (Fig. 9) that could not be interpreted as "bands".

To estimate the molecular weight of the hemolysin, a portion was passed through a standardized Sephadex G-100 column. Figure 10 shows the results of column calibration and the extrapolation of the hemolysin's molecular weight which was approximately 55,000.

Table 8 shows the results of the amino acid analysis of the hemolysin. The presence of carbohydrate in the protein preparation prohibited the quantitation of leucine and tyrosine. Also given is the amino acid composition of toxin from V. parahaemolyticus WP-1 (data recalculated from Honda et al. (21)).

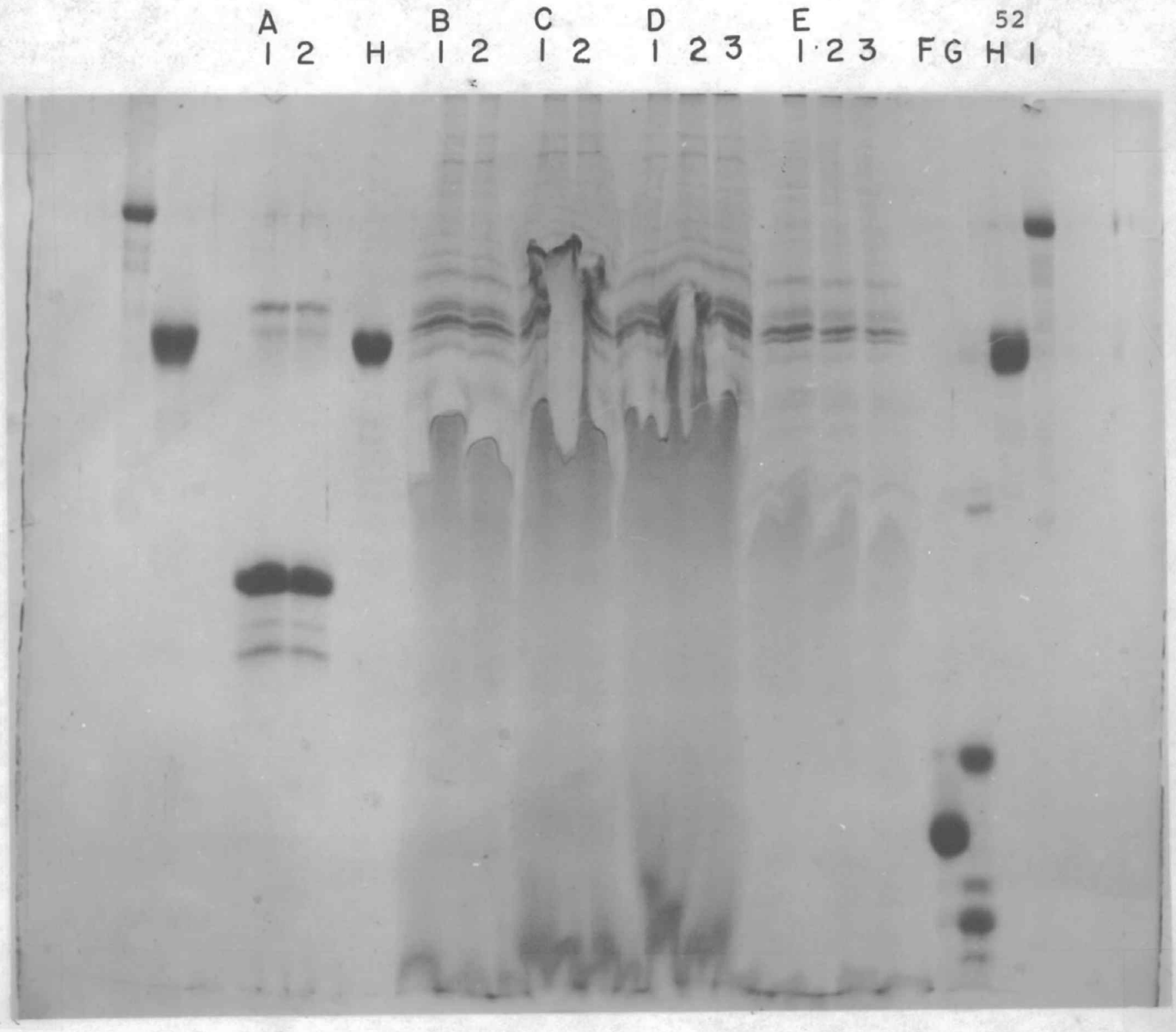

Figure 9. SDS polyacrylamide gel electrophoresis of the hemolysin in 0.02 M and 0.25 M phosphate buffer. The wells contain: A 1-2 = 10 µl and 15 µl of vibriolysin; B 1-2 = 20 µl and 30 µl of hemolysin (preparation #5) in 0.25 M phosphate buffer; C 1-2 = 20 µl and 30 µl of hemolysin (preparation #5) in 0.02 M phosphate buffer; D 1-3 = 10 µl, 20 µl and 30 µl hemolysin (preparation #6) in 0.02 M phosphate buffer; E 1-3 = 10 µl, 20 µl and 30 µl hemolysin (preparation #6) in 0.25 M phosphate buffer; F = 7 µl cytochrome c; G = 7 µl α chymotrypsinogen A; H = 7 µl aldolase; and I = 5 µl BSA.

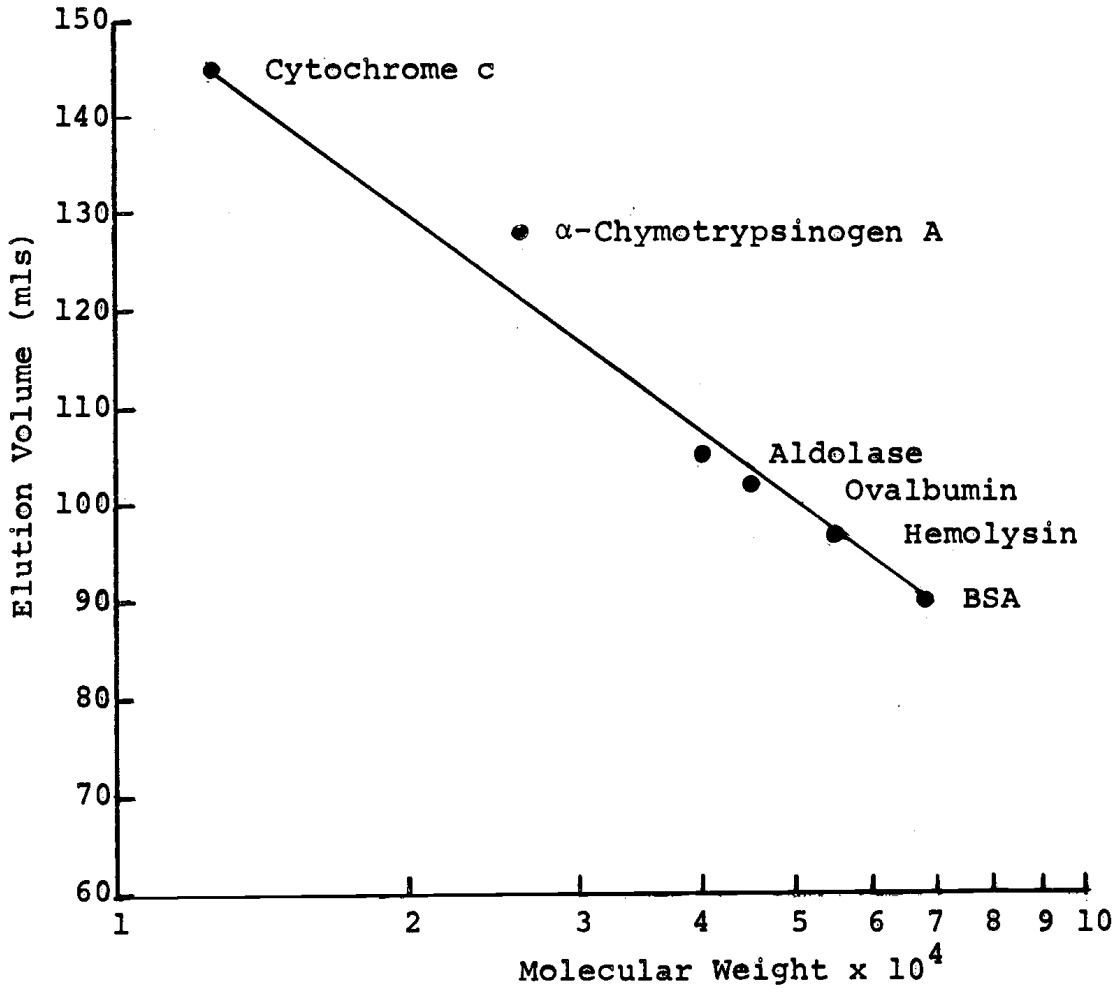

Figure 10. Molecular weight estimation of the hemolysin by Sephadex G-100. The column was calibrated with protein standards and the molecular weight of the hemolysin was determined.

Table 8. Amino acid analysis of hemolysin from isolate 123-S1.

Amino acid	Weight % *V. parahaemolyticus* WP-1 toxin[1]	Isolate 123-S1 hemolysin[2]
Aspartic acid	12.6	10.0
Glutamic acid	11.0	8.2
Valine	10.8	4.7
Tyrosine	9.2	--
Phenylalanine	8.8	7.2
Lysine	7.1	4.9
Serine	7.0	4.5
Threonine	5.7	3.9
Proline	4.1	1.9
Leucine	4.0	--
Isoleucine	3.2	4.0
Alanine	3.0	4.9
Glycine	2.8	6.6
Histidine	2.7	2.4
Arginine	2.7	3.7
Methionine	2.3	1.5

[1] Data recalculated from Honda *et al.* (21).

[2] Tyrosine and leucine could not be determined due to presence of carbohydrate.

DISCUSSION

There are many unanswered questions about the origin, production, and activity of the hemolytic exotoxin of V. parahaemolyticus. The reason why only some of the strains of V. parahaemolyticus are pathogenic is not understood. The transient nature of the pathogenic characteristic is also puzzling. It has been suggested (3, 7, 8, 18) that the genes responsible for hemolytic exotoxin are not inherent to the organism's genome but associated with bacteriophages or plasmids. Thus the necessary genetic material could be acquired through genetic exchange in the natural environment. The origin or natural reservoir for the production of the Kanagawa hemolysin and pathogenicity of V. parahaemolyticus may be another marine vibrio with a wide temperature growth range (7).

Hemolytic marine vibrios distinct from V. parahaemolyticus have been previously described (5, 55). However, there have been no significant reports concerning the incidence or characteristics of the Kanagawa positive Vibrio spp. other than V. parahaemolyticus.

The objective of this research was to isolate Kanagawa positive psychrotrophic marine vibrios and compare one hemolysin with the hemolytic exotoxin of V. parahaemolyticus. These psychrotrophic vibrios were found to differ biochemically from V. parahaemolyticus.

After sample enrichment and culture isolation the unwanted organisms were screened out by biochemical examination. Isolates were simultaneously tested for their gram reaction and morphology, the Kanagawa reaction and selected biochemical responses. Subsequent biochemical examination was done on all organisms that were gram negative rods, motile, oxidase positive, required sodium chloride for growth and were fermentative on TSI agar without gas or hydrogen sulfide production.

Approximately 11% of the 235 vibrio strains isolated were Kanagawa positive. These Kanagawa positive vibrios differed from V. parahaemolyticus in their amino acid decarboxylase reactions, growth at 42°C, and cellobiose and sucrose fermentation. A single isolate was chosen for further study based on the production of a high level of hemolysin. The organism selected, isolate 123-S1, may possibly be either a strain of V. anguillarum or a member of the unclassified vibrio group.

The hemolytic activity of a crude hemolysin preparation was destroyed by treatment with pronase or protease indicating its proteinaceous nature. Therefore a combination of methods used in protein isolation were employed.

Multistep protein purification protocols often begin with the isolation of the desired substance from solution by precipitation. Ammonium sulfate, commonly employed for this purpose, was used because it did not irreversibly inactivate the hemolysin. Also, the resulting protein

precipitates were then easily stored at low temperatures without significant loss of activity.

The hemolysin was released from the DEAE-cellulose column with approximately 0.25 M to 0.35 M NaCl (in 0.02 M phosphate buffer). After elution from the DEAE-cellulose column, the presence of 0.25 M salt in all buffers and solutions was required for hemolysin stability. However, this salt effect was reversible. That is, hemolysin inactivated by a low salt concentration had activity restored by the addition of 0.25 M sodium chloride or phosphate buffer.

Since the protein and hemolytic peaks were not completely coincident further separation of hemolysin from other protein compounds was attempted using gel filtration. However, the non-hemolytic protein also present in the hemolysin fraction did not appear to be different enough to allow for a complete protein separation by Sephadex.

Typical results on partial purification were presented. Generally, one objective of protein purification is to concentrate the protein and to increase the specific activity of the preparation. The reduction of total protein and total activity seen was typical. During handling and transfer the amount of total protein (hemolytic and non-hemolytic) decreased. Also, as purification proceeded, non-hemolytic proteins were at least partially removed. The total activity, or total number of hemolytic units, also decreased with total protein.

Ideally, specific activity increases throughout the purification process. The data showed that specific activity increased until it was eluted off the anion exchange column. After this point there was a slight drop, approximately 8%, in the specific activity of the hemolysin preparation. This loss was probably attributed to small individual losses that occurred during reprecipitation with ammonium sulfate, dialysis and Sephadex filtration.

The Sephadex eluate was preferred for use in further hemolysin characterization. Regardless of the lowered specific activity, this preparation contained less non-hemolytic protein and possibly less other contaminating substances. Partial characterization of the hemolysin was done in an effort to compare its properties to those of the exotoxin from V. parahaemolyticus WP-1.

The temperature range of activity was determined. The hemolysin's optimal temperature for activity was 37°C, a temperature at which 123-S1 will not grow. A similar activity range has not been reported for the purified toxin from a Kanagawa positive V. parahaemolyticus. However, it has been generally assumed that the hemolytic toxin is not active at low temperatures.

The heat sensitivity pattern of a toxin is an identifying characteristic. A discontinuous effect of activity to increasing temperature, or the Arrhenius effect, is known to occur with several bacterial toxins. However, the

hemolysin, in the absence of rabbit erythrocytes was rapidly heat inactivated at 30°C or higher. There was no evidence of an inactivating factor present in the hemolysin preparation.

Vibriolysin preparations of similar purity, usually contaminated with an inactivating factor, had a disproportionate effect to heating (32, 39, 41, 42, 46). Hemolytic activity was destroyed at 60°C but was present after heating at 90°-100°C.

The exotoxin of V. parahaemolyticus WP-1 has been shown to be a pure protein (21). The partially purified hemolysin isolated from 123-S1 was protein or a protein containing compound since its activity was diminished by proteolytic enzymes. The carbohydrate also present in the preparation interfered with some of the characterization tests.

Certain types of gangliosides that naturally occur on many cell surfaces, serve as receptor sites for some hemolysins and toxins. Several bacterial toxins are known to be inactivated by preincubation with gangliosides (24).

The hemolysin's activity seemed to be partially affected by preincubation with a mixture of gangliosides. There was only a partial reduction in activity (35%) upon the addition of 1 mg/ml of a ganglioside mixture. The correlation between the degree of hemolysis and the ganglioside concentration was somewhat vague. Before a definite

interdependent relationship between these compounds can be suggested, further examination with many types of gangliosides would be required.

There are several factors to consider in the interpretation of these results. The presence of carbohydrates in the hemolysin preparation may have acted as a protective colloid. This could have masked a fuller effect that gangliosides might actually have on the hemolysin if it were present as a pure protein. Since gangliosides have a marked tendency to combine with many large organic compounds, any contaminating material may have interfered with this test. Also it was noted that toxins present in low concentrations tend to be nonspecifically and only slightly affected by gangliosides (24). The effects of gangliosides are not fully understood, but it is known that the chemical and/or physical environment dictates the ability of these lipids to bind various bacterial toxins (24).

It has been reported (21, 48, 49) that a ganglioside mixture completely inhibited the hemolytic and lethal character of V. parahaemolyticus toxin. Specifically, 50 µg of toxin was inactivated by 0.4 mg of ganglioside (21).

The reactivity of vibrio hemolysins to horse erythrocytes has been an important characteristic. Horse red blood cells apparently do not contain the same types of gangliosides, which serve as surface receptors for

hemolysins, as those found on the more susceptible rabbit erythrocytes (48). The strains of Kanagawa positive V. parahaemolyticus tested (WP-1 and 4750) did not hemolyze horse red blood cells (31, 48, 55).

A correlation exists between the types of gangliosides that will bind and therefore inactivate a hemolysin, and the types of erythrocytes that are susceptible to hemolytic action. There is a gradient of lytic activity that the hemolytic exotoxin of V. parahaemolyticus has against red blood cells of different animals (55). Susceptible erythrocytes contain, on their membrane surface, a critical amount of ganglioside that binds hemolysin. A ganglioside extraction from the red blood cells of horses did not contain the necessary classes of gangliosides for binding this exotoxin (48).

Hemolysin from 123-S1 showed a lower reactivity to horse erythrocytes. One hemolytic unit incubated with horse cells produced only half of the hemolysis expressed with rabbit erythrocytes. This intermediate reaction might be correlated with the lowered hemolysis seen after 1.0 mg/ml ganglioside was preincubated with 50 µg of 123-S1 hemolysin.

The hemolysin from 123-S1 was tested for antigenic similarity to purified toxin (vibriolysin) from V. parahaemolyticus WP-1 by a standard two dimensional diffusion test. The homologous antibody (antivibriolysin) was used to check for precipitation with the hemolysin. A variety

of different concentrations of hemolysin and antivibriolysin were tested together but no observable precipitation occurred.

Results do not support an antigenic relationship between the hemolysin of 123-S1 and V. parahaemolyticus WP-1. However, Zen-Yoji et al. (56) demonstrated that not all of the hemolysins produced by Kanagawa positive strains serologically cross react with each other. Only 2 out of 3 strains of Kanagawa positive V. parahaemolyticus isolates produced hemolysins that would react with an antibody prepared against a hemolysin from a fourth strain.

The homogeneity of a protein preparation, the presence of subunits and the molecular weight can often be determined with SDS polyacrylamide gel electrophoresis. After concentration, the hemolysin was applied to this type of gel to determine its electrophoretic pattern. Honda et al. (21) reported that his preparation of exotoxin produced one band upon electrophoresis, inferring the presence of a single protein. A sample of the lyophilized exotoxin from Japan (courtesy Y. Takeda) was tested on SDS polyacrylamide gel electrophoresis and five distinct bands were evident.

The banding pattern produced by a hemolysin preparation from 123-S1 revealed three major bands. Their approximate molecular weight was 55,000, 48,000, and 46,000. Several minor bands and some impurities were also observed. Because of these results the information gained from this experiment

was limited. However, some of the major bands of 123-S1 hemolysin and V. parahaemolyticus toxin occupied similar positions.

The hemolysin appeared to require the presence of 0.25 M phosphate buffer in order to form discrete bands. Although SDS tends to disrupt protein structure, a high salt concentration may be necessary to maintain some type of conformation to prevent irregular and random aggregation. The banding of protein standards electrophoresed with 0.02 M or 0.25 M phosphate buffer were not significantly different.

Molecular exclusion chromatography is a simple way to approximate the molecular weight of a protein in an unpurified state. Sephadex G-100 has been shown to be the gel of choice for many proteins (1). The molecular weight of purified exotoxin was approximately 42,000 (21). The molecular weight of the hemolysin from 123-S1 was estimated to be 55,000.

The presence of carbohydrate in the hemolysin preparation may interfer with the molecular weight estimation by this technique. Differences of this measure might also be partially due to other factors. Although the same protein markers were used in both experiments, the use of different volumes of these standards can produce different standard curves. Some proteins, such as aldolase and α-chymotrypsinogen A, have a concentration

dependent elution volume (1). If the concentration is low, the proteins dissociate into smaller subunits and elute differently than when in their native conformation (1). Honda et al. (21) did not include information on the volume or concentration of the protein markers, therefore it is not possible to evaluate these considerations.

The molecular weight sizing of the major protein bands on SDS polyacrylamide gel electrophoresis was understandably varied from the approximation on Sephadex. Polyacrylamide gel electrophoresis is a more accurate technique for molecular weight sizing (53), it reveals some of the protein components and their corresponding molecular weights. Some type of combined molecular weight value is obtained with Sephadex gel filtration.

The amino acid composition of a protein has been used for characterization purposes and in the explanation of other properties. Both the exotoxin of V. parahaemolyticus WP-1 and hemolysin from 123-S1 had a high weight percentage of the acidic amino acids, aspartic and glutamic acid. Also, both were low in the basic amino acids arginine and histidine. The sulfur amino acid, methionine, appeared in the lowest weight percentage in both of the samples.

A large amount of information on the nature of the Kanagawa reaction has been published since its discovery. However, most of the work was done with a substance derived from what is now considered to be an inferior purification

scheme. Some of the older reports on the properties of exotoxin from V. parahaemolyticus may be inaccurate. For example, there are no published reports that test the thermolability of the toxin obtained from Honda et al.'s (21) newer purification scheme. All of the physiochemical properties reported by Honda et al. have yet to be verified by others.

Preliminary studies by Zen-Yoji et al. (56) demonstrated that not all the strains of Kanagawa positive V. parahaemolyticus appear to produce an identical hemolysin. Unfortunately there has been intense biochemical studies on toxin from only one strain of V. parahaemolyticus. The properties reported from this type of work may not apply to the hemolytic exotoxins produced from all Kanagawa positive strains. It may not be possible to generalize about exotoxin characteristics from V. parahaemolyticus. The hemolytic exotoxins may be strain dependent and therefore a number of slightly different toxins may exist.

Pathogenesis may require many extracellular products for expression. Some of these products may be proteases that destroy tissues and immunoglobulin, hemolysins and related substances that effect various cells, and toxins that destroy leukocytes and damage tissues whose functions are vital to the host (27). Therefore, a complete understanding of disease production by any bacterial species might be achieved only when all of these factors are

considered. This is further substantiated by the uncertainty of whether the purified exotoxin of V. parahaemolyticus is itself even enteropathogenic.

It was discovered that the purified exotoxin was not effective in causing dilation in rabbit ileal loops (20). This may reflect the loss of some of the toxin's components that are necessary for activity. Perhaps the inactivating factor, and/or other substances are needed for maximum effectiveness under in situ conditions. If biologically active exotoxin of V. parahaemolyticus exists naturally as aggregated subunits, as does V. cholerae toxin, the inactivating factor may be part of a multicomponent toxin moiety.

The genes for toxin production in bacteria have been found on chromosomes, temperate bacteriophage DNA and on plasmids (29). The location of toxin producing genes on extrachromosomal elements such as temperate phage and plasmids, greatly increases the range of bacterial strains to which they can be distributed (29).

Since it has been shown (6, 8) that V. parahaemolyticus and a high number of many other vibrio species can coexist in the intestine of inshore marine animals, it is reasonable to consider that these species may be genetically interacting. These interactions might include a phage or plasmid mediated transfer of genetic material. Baross et al. (6, 8) has reported that there was a high frequency of phage present in molluscan shellfish capable of lysing V.

parahaemolyticus. The presence of the phage was correlated with the incidence of mesophilic vibrio species. It was suggested that the origin of these phages may be a vibrio species other than V. parahaemolyticus. It is possible then that V. parahaemolyticus may be sharing bacteriophage and thus genetic material with other currently unclassified vibrio species (6, 7), If such genetic exchange does occur, it might be in this manner that V. parahaemolyticus acquires the appropriate genetic material from another vibrio species for the expression of the Kanagawa phenomena and pathogenicity (7).

The majority of known tox genes, in other pathogenic bacteria, are apparently located on extrachromosomal elements (29). This strongly suggests that more emphasis should be placed on a genetic approach to studies concerning the pathogenicity of V. parahaemolyticus. Areas that are especially worthy of future research include the unclassified hemolytic marine vibrios and the transducing bacteriophage often isolated in the estuary environment.

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