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Purification and Characterization of the Soluble Hemagglutinin (Cholera Lectin) Produced by *Vibrio cholerae*

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The soluble hemagglutinin (HA) (cholera lectin) produced by *Vibrio cholerae* strain CA401 was purified to apparent homogeneity by a sequence of ammonium sulfate fractionation, gel filtration, and preparative isoelectric focusing. Soluble HA activity was found to focus at three different pIs, 6.3, 5.3, and 4.7. Each of the factors migrated as a large-molecular-weight protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis under normal conditions, and each, upon heating in sodium dodecyl sulfate was found to dissociate into 32,000-molecular-weight subunits. Treating the samples with a reducing agent did not affect their mobility. Each gave a reaction of immunological identity with antiserum prepared against the others. Thus, there are apparently three distinct pH isotypes of soluble HA which exist as noncovalently associated polymers of 32,000-molecular-weight subunits. Electron microscopy of purified preparations revealed long filamentous polymers. The molecule does not stain as a glycoprotein; it is hydrophobic; it is inactivated during incubation at 25, 37, or 60°C; and it has significant protease activity. The protease activity likewise focused at pH values of 6.3 and 5.3 to 4.7, and it was inhibited by antiserum against the HA. However, whereas the HA is active at 4°C, the protease is not. The soluble HA is, therefore, a bifunctional molecule capable of mediating hemagglutination and proteolysis. Its amino acid composition is reported. Fab fragments of antibody against the purified HA inhibited attachment of heterologous serotype-biotype *V. cholerae* to infant rabbit small bowel.

In a previous paper (7), we described the presence of four kinds of hemagglutinins (HAs) in *Vibrio cholerae*: a cell-associated mannose-sensitive HA which is characteristic of the El Tor biotype, a cell-associated L-fucose-inhibitable HA, another cell-associated HA which appeared transiently in late log-phase cultures, and a soluble HA which is found in the supernatant of the late log phase of all of the cultures examined and which is insensitive to inhibition by any monosaccharides tested. Because a previous report (4) indicated that the soluble HA (called cholera lectin) from a classical biotype Inaba serotype cholera vibrio was capable of blocking attachment of El Tor biotype Ogawa serotype cholera vibrios to rabbit ileum and other tissues and because soluble HA is produced by all strains examined (7), the molecule may be important in the pathogenesis and immunology of *V. cholerae* infection. This communication describes the purification and molecular characterization of the soluble HA.

(This work constitutes a portion of the dissertation of L.F.H., submitted in partial fulfillment of the requirements for the Ph.D. degree, University of Texas Health Science Center at Dallas, of which the majority was performed in absentia at the University of Missouri at Columbia.)

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MATERIALS AND METHODS

Bacterial strains. *V. cholerae* strain CA401 is a classical biotype, Inaba serotype originally isolated in Calcutta in 1953 by C. E. Lankford. It and other *V. cholerae* strains used have been described previously (4, 7, 9).

HA assay. The techniques for microtiter quantitation of HA activity were as described (7). Responsive and nonresponsive chicken erythrocytes (7) used for monitoring HA activity throughout the purification scheme, were obtained from individual chickens from an inbred line of White Leghorn chickens at the University of Missouri Department of Poultry Husbandry.

Purification of soluble HA. *V. cholerae* CA401 was grown confluently on four petri plates of meat extract agar overnight at 37°C. Growth was harvested with sterile tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) and inoculated into 9.5 liters of TSB in each of four fermentor jars. Fermentors (New Brunswick Scientific, Inc., Edison, N.J.), to which were added 3 ml of antifoam (antifoam B, Sigma Chemical Co., St. Louis, Mo.), each were incubated at 30°C with vigorous aeration for 20 h. Merthiolate, 1 g, was

added, and the cells were removed by continuous-flow centrifugation. The supernatant was concentrated, in the cold, on PM10 (10,000 molecular weight exclusion) filter membranes (Amicon Corp., Lexington, Mass.) to approximately 500 ml. Any bacterial cells which were not previously removed by centrifugation were sedimented for 10 min at 6,500 × *g*. The cell-free concentrated supernatant was then treated with 25% saturated ammonium sulfate and, after removal of the precipitate by centrifugation, the supernatant was further raised to 50% saturation with ammonium sulfate. The 25 to 50% $(NH_4)_2SO_4$ insoluble material was resuspended in approximately 150 ml of Tris-EDTA-azide-NaCl (TEAN) buffer (5), and dialyzed through two changes to TEAN buffer (1:30, dialysand-dialysate). Insoluble material was removed by centrifugation at 19,000 × *g* for 15 min. This concentrated supernatant was designated crude HA.

Crude HA was subjected to gel filtration chromatography on a Sephadex G75 column (5 by 140 cm) eluted with TEAN at 4°C. Fractions with HA activity were pooled and concentrated to 3 ml on a PM10 membrane. Samples, 8 mg of protein in 1 ml, were subjected to isoelectric focusing (IEF) with a pH gradient from pH 4 to 9 (Biolyte 4/9; Bio-Rad Laboratories, Richmond, Calif.) in a Sephadex gel matrix (Ultrodex; LKB Instruments, Inc., Rockville, Md.) with an LKB Multiphore IEF unit. IEF was carried out for 16 h, at 4°C, beginning with 8 W of constant power. After the material had focused, a print of the gel was taken by overlaying the bed with a strip of Whatman filter paper for 2 min. The paper was dried at 250°F (121°C) for 10 min, and then stained for protein with 0.1% Coomassie brilliant blue R in acetate-methanol-water (10:45:45) for 15 min. The gel bed was partitioned, and the Ultrodex was scooped from each section with a spatula. Material was eluted through glass wool-plugged Pasteur pipettes with 2 ml of phosphate-buffered saline (PBS). All operations and storage were at 4°C.

SDS-PAGE. We used a modified Laemmli (10) system for both slab and disc gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide slab gels (12%) with a 3% stacking gel were electrophoresed approximately 5 h at 25 mA constant current per slab with a constant flow of ice water as coolant. Slab and tube gels were stained with 0.1% Coomassie brilliant blue R (Sigma) in acetate-methanol-water (7:43:50) and destained in the same solution without the dye. Non-SDS 5% tube gels (5 by 100 mm) were run and stained similarly. The current was 3 mA per tube.

Periodic acid-Schiff staining for carbohydrate. The periodic acid-Schiff staining procedure was adapted from Zacharius et al. (26).

Protein determination. All reported protein determinations were by the method of Bradford (2).

Antisera. Young adult New Zealand white rabbits were inoculated subcutaneously in multiple sites with 100 μg of immunogen in Freund complete adjuvant (Difco). Animals were bled from the marginal ear vein from 2 to 6 weeks after vaccination. Fab fragments were prepared by digestion of ammonium sulfate-precipitated immunoglobulin by treatment with mercuripapain (Sigma) followed by iodoacetamide by the method of Porter (16). The Fab fragments, which retained their ability to inhibit HA, exhibited no detectable vibriocidal or agglutinating activity and, in contrast to the parent immune serum, gave no precipitin reaction with crude or purified soluble HA.

Amino acid composition. The amino acid composition of the soluble HA purified from CA401 was determined by C. W. Gehrke, College of Agriculture, University of Missouri, Columbia. Their laboratory used an ion-exchange methodology modified from Benson and Patterson (1).

Electron microscopy. Transmission electron micrographs of the soluble pI 6.3 HA purified from CA401 were prepared by D. Sherman, Department of Anatomy, University of Missouri, Columbia. A small drop of the purified HA (500 μg/ml) was placed on a 200-mesh carbon-coated Formvar grid for 1 min. Excess material was removed with filter paper. A drop of 2% uranyl acetate solution was applied to the grid for 2 min, and the excess was removed with filter paper. The preparations were then air dried. Samples were examined with a Phillips 300 transmission electron microscope at 80 kV and 20,000 magnification. Photographic negatives were enlarged approximately three times, giving a final magnification of ×60,000.

Azoalbumin protease assay. The azoalbumin protease assay was modified from Tomarelli et al. (21). Azoalbumin (Sigma), 5 mg/ml in TEAN buffer, was the enzyme substrate. We mixed 100 μl of substrate (0.5 mg) with 50 μl of dilutions of unknown samples. Tubes were incubated at 37°C (or other temperatures for temperature optimum determinations) for 1 h. We then added 3 ml of 3% trichloroacetic acid to precipitate-undigested substrate. After 20 min at room temperature, the tubes were centrifuged at 900 × *g*. We decanted 2 ml of the supernatant and added it to 2 ml of 0.5 M NaOH. The neutralized samples were agitated briefly, and the optical density at 440 nm was determined with a Spectronic 20. A standard curve of digested substrate was generated by incubating different concentrations of the azoalbumin substrate with 1 mg of pronase (Sigma) per ml for 1 h to effect complete digestion. One unit of protease activity by this assay was defined as that which will digest 20% of the substrate (i.e., 0.1 mg) in 1 h.

The dimethyl-casein method of Lin et al. (12) was also used. That assay measures the number of free amino groups generated. The results paralleled those obtained in the azoalbumin assay.

Inhibition of binding. Vibrios (strain 3083) (14) were grown overnight at 30°C on a rotary shaker in 2 ml of syncase medium (in 25- by 150-mm tubes) plus 2 μCi of [D-^{14}C]glucose (240 mCi/mmol; Schwarz/Mann, Orangeburg, N.Y.) for 5 to 6 h and washed three times in 0.1% peptone–0.85% NaCl. Ileum was removed from infant (10-day-old) New Zealand white rabbits, opened longitudinally, and rinsed in saline. Disks were then cut with an 8-mm-diameter cork borer. One set of disks was pretreated for 15 min in purified HA (70 μg/ml). The tissue slices were then added to ^{14}C-labeled vibrios (approximately 5×10^9) in peptone-NaCl or peptone-NaCl containing Fab fragments of anti-HA serum (and preimmune serum treated similarly). These preparations contained similar amounts (35 to 40 mg/ml) of protein. After 20 min at room temperature (preliminary experiments indicated that the vibrios attached as well at room temperature as at 37°C), the tissue slices were removed, rinsed, and transferred to scintillation vials for digestion (13). After digestion and cooling, 3 ml of Aquasol 2 (New England Nuclear

TABLE 1. Purification of CA401-soluble HA[a]

Preparation	HA titer	Vol (ml)	HA U (titer × vol)	Protein (mg/ml)	Sp act (HA U/mg of protein)	% Recovery
Cell-free culture supernatant	128	35,000	4,480,000	0.13	984	100
Culture supernatant concentrated 50-fold	4,840	820	3,968,800	3.8	1,273	88
Crude[b]	5,120	220	1,126,400	9.0	569	25
Sephadex G75 region 1[c]	64	1,200	76,800	ND[d]	ND	1.7
Sephadex G75 region 2[e]	3,072	30	92,160	8.0	384	2.1
IEF pI 6.3	256	15	3,840	0.56	457	0.09
IEF pI 5.3	32	15	430	0.26	123	0.01
IEF pI 4.7	64	15	960	0.58	110	0.02

[a] Values are taken from four fermentors of CA401 grown in TSB for 20 h processed as described in Materials and Methods. Values are adjusted to reflect the yield if all 35 liters were carried through the entire purification protocol.

[b] Ammonium sulfate cut 25 to 50%.

[c] See Fig. 1.

[d] ND, Not done.

[e] Pool of second region of activity, pooled and concentrated over a PM10 membrane.

Corp., Boston, Mass.) scintillation cocktail was added, and the samples were counted for 1 min in a Beckman model LS7000 scintillation counter. Experiments were performed in quadruplicate, and statistical analysis was performed by the Whitney-Mann U test (27). In one experiment, ligated ileal loops in an adult rabbit (4) were pretreated for 10 min with 1, 10, or 100 μg of purified HA (with bovine serum albumin in control loops). ^{14}C-labeled vibrios were added, and after 3 h, the rabbit was sacrificed, and the loops were opened, cut into fifths, rinsed, digested, and counted as above.

RESULTS

After numerous attempts, the soluble HA produced by CA401 in TSB fermentor cultures was purified to apparent homogeneity by a sequence of differential salting out with ammonium sulfate, gel filtration, and preparative IEF.

Approximately 75% of the activity was lost during ammonium sulfate precipitation (Table 1). Gel filtration on Sephadex G75 resulted in further (>90%) losses of activity. The activity which could be recovered appeared in a broad area after the ascending edge of the first major peak (Fig. 1). The activity associated with the void volume peak (region 1) accounted for about 50% and region 2 accounted for the rest of the recoverable activity.

Because of the large amount of lipopolysaccharide and other contaminating materials presumed to be associated with it, the material in region 1 was not processed further. The second region was pooled and concentrated over a PM10 membrane. The concentrated material from region 2 was further separated by preparative IEF on a gradient from pH 4 to 9. The distribution of protein after this procedure is shown in Fig. 2. Assay of fractions collected throughout the gradient revealed three major regions of HA activity. The majority of recoverable activity focused at pH 6.3. The other two major regions of activity focused at pH values of 5.3 and 4.7 and had other contaminating proteins in close proximity. HA activity was detected in these three regions on repetitive IEF runs of the same pool. Material from subsequent fermentor cultures of CA401, prepared similarly, also gave three regions of activity upon IEF of Sephadex G75-chromatographed material. When an identical purification protocol was used to purify the soluble HA from an El Tor biotype vibrio, strain 3083, HA activity focused at pH 6.3 and 5.3.

The HA at all stages of purification was active only on responder chicken erythrocytes and was not active on nonresponder chicken erythrocytes (7). The three separate IEF activities also exhibited this limited erythrocyte spectrum.

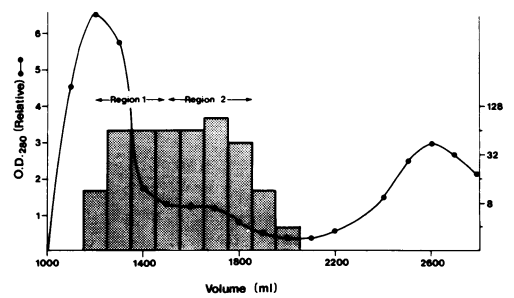

FIG. 1. Sephadex G75 (140 by 5 cm) column gel filtration of CA401-soluble HA (ammonium sulfate precipitate). Samples (50 ml) were applied and chromatographed at 50 ml/h in TEAN buffer; 10-ml fractions were collected and assayed for HA activity. Region 1 was discarded. Region 2 was pooled, concentrated over a PM10 ultrafiltration membrane, and subjected to further purification.

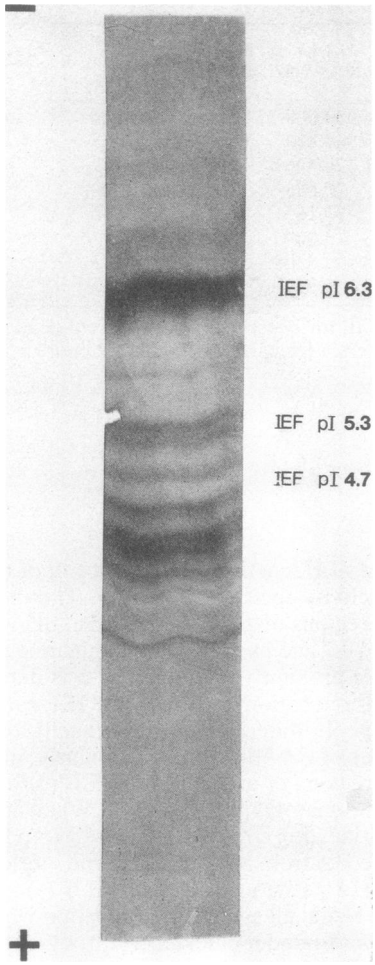

FIG. 2. Preparative IEF of G75-chromatographed soluble HA. Sephadex G75-purified material (8 mg) was focused in a 50-ml Ultrodex (LKB) matrix with a pH 4 to 9 gradient. Constant power (8 W) was applied for 20 h. A Whatman filter paper print of the run was stained for protein with Coomassie brilliant blue R.

The three regions of HA activity from the IEF separation were analyzed on SDS-PAGE (Fig. 3). All three regions electrophoresed identically, although IEF pIs 5.3 and 4.7 had minor contaminating bands. It can be noted that, unheated, all preparations barely entered the gel. Reduction had no effect on the mobility, suggesting no interchain disulfide bonds. However, upon treating the three preparations at 100°C for 2 min (either reduced or unreduced), the high-molecular-weight form dissociated into identically sized subunits. Comparison of the subunits with appropriate standards revealed a molecular weight of approximately 32,000.

The stability of the HA activity at various temperatures was evaluated by incubating crude

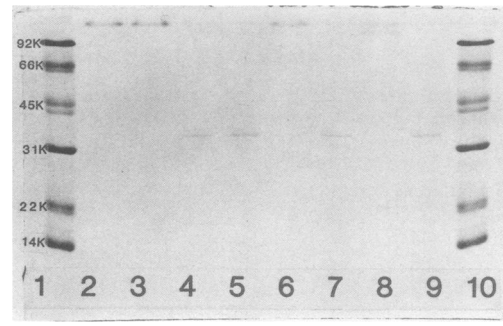

FIG. 3. SDS-PAGE (12% acrylamide) of IEF-purified soluble HA from strain CA401. Samples (15 μg) were electrophoresed at 25 mA per slab for 4.5 h, then stained for protein. IEF pIs 6.3, 5.3, and 4.7 show a large-molecular-weight multimer which dissociates into 32,000-molecular-weight subunits upon heating. Lane 1, molecular weight markers, 92, 66, 45, 31, 22, and 14 kilodaltons, respectively; lane 2, CA401 pI 6.3; lane 3, CA401 pI 6.3 reduced with 2-mercaptoethanol; lane 4, CA401 pI 6.3 boiled for 2 min in SDS; lane 5, CA401 pI 6.3 reduced and heated; lane 6, CA401 pI 5.3; lane 7, CA401 pI 5.3 heated; lane 8, CA401 pI 4.7; lane 9, CA401 pI 4.7 heated; and lane 10, molecular weight markers.

soluble HA at 60, 37, 25, and 4°C (Fig. 4). HA activity was very sensitive to inactivation at 60°C, being entirely abrogated within 1 h. Activity was also sensitive to incubation at 37 and 25°C, although it was relatively stable at 4°C. Such rapid degeneration of HA activity at 37 and 25°C was suggestive of enzymatic digestion of the molecule. Therefore, protease activity was monitored sequentially during purification of the HA.

Two fermentors of CA401 supernatant were

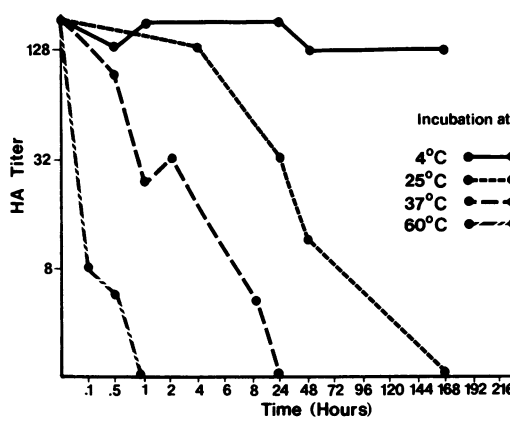

FIG. 4. Temperature stability of ammonium sulfate-precipitated soluble HA incubated at 4, 25, 37, and 60°C. Samples were incubated at the four temperatures and assayed for HA activity periodically.

TABLE 2. Specific activities of soluble HA and protease from *V. cholerae* CA401[a]

Preparation	Vol (ml)	Protein (mg/ml)	HA titer	Sp act HA U/mg	Protease[b]		Sp act of HA/ protease ratio
					U	Sp act (U/mg)	
Cell-free culture supernatant	19,000	0.07	96	1,371	5	71	19.3
Culture supernatant concentrated 40-fold[c]	525	1.6	1,280	800	135	84	9.5
Salt out 25 to 50%	56	5.6	12,800	2,285	1,000	178	12.8
Sephadex G75 region 2[d]	4.5	4.35	800	184	300	69	2.6
IEF pI 6.3	9	0.12	8	67	40	333	0.2

[a] Two fermenters of CA401 were grown for 20 h at 30°C in TSB. The protocol devised for purification of soluble HA (described earlier) was followed. Values reflect the purification of the entire preparation through the final step.

[b] One unit of protease, concentration in 50 μl which will digest 0.1 mg of azoalbumin per h. This corresponds with the release of 3×10^{-4} M NH_3 per h as determined by the dimethyl-casein assay described in Materials and Methods.

[c] The supernatant was concentrated over a PM10 (molecular exclusion, 10,000) membrane.

[d] For region 2 of HA activity, see Fig. 1.

subjected to the purification protocol. HA and protease activity were monitored at each stage (Table 2). If the HA and protease are one molecule, their specific activities should increase (or decrease) in parallel at each purification step. This is reported in the last column as the ratio of the HA to the protease specific activities. In the cell-free supernatant, the ratio was 19.3 HA U/protease U. However, the purified material had a ratio of 0.2 HA U/protease U. The most dramatic changes in this ratio occurred after gel filtration and after IEF when the specific activity of the HA fell drastically. These results suggested that the HA and protease activities might be mediated by different molecules.

However, analysis of protease activity after preparative IEF revealed major regions of activity which focused at pH 6.3 and in a broad area from 5.3 to 4.7 (Fig. 5). This corresponded almost exactly with the pIs of the HA activity. It would be very unlikely for two different molecules to have the identical three pIs.

Additionally, both activities could be eluted from unstained non-SDS disc gels only in the area corresponding with the single protein staining band of the pI 6.3 material. This was also demonstrated (Fig. 6) by running parallel non-dissociating gels, staining one, and embedding the other in 1.5% skim milk agar (20). As can be seen, the single protein staining band coincides with the halo of proteolytic hydrolysis of the casein substrate (20). These data clearly associate both HA and protease activity with the same molecule. Further, specific antibody against the HA, which gives a single band of immunoprecipitate with both crude and purified HA (and which, as described below, inhibits attachment of cholera vibrios to intestinal epithelium), inhibits the protease activity (Fig. 6). The molecule thus appears to be bifunctional. Further studies (Table 3), however, indicated that the two activities have different temperature requirements. HA titers were relatively constant when assayed over the range from 4 to 37°C. Protease activity, on the other hand, was more dependent on the incubation temperature: 100-fold differences in protease activity were observed between 4 and 25°C incubation.

Electron microscopy of the pI 6.3 material revealed that the purified HA had aggregated into long filaments (Fig. 7). Its amino acid composition is summarized in Table 4.

The specific antiserum raised to purified soluble HA was used to examine the soluble HA from several strains of *V. cholerae* (Fig. 8).

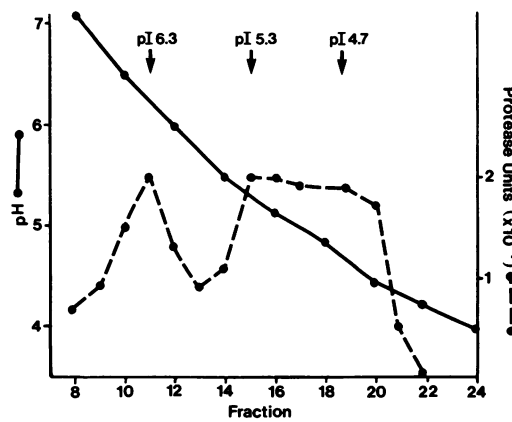

FIG. 5. Protease activity and pH profile of preparative IEF (pH 4 to 9) of Sephadex G75-chromatographed soluble HA from CA401. The pH of the gel bed was determined after focusing, then material was eluted from the Ultrodex matrix with PBS and assayed for HA and protease activity. HA activity was also found to focus at pH values of 6.3, 5.3, to 4.7.

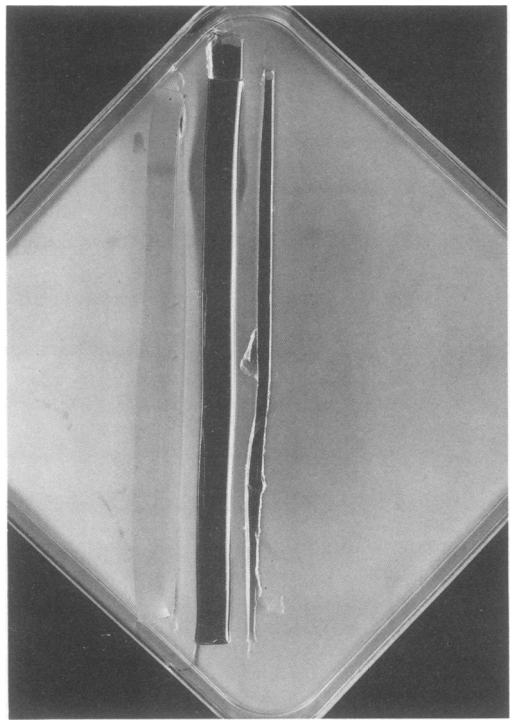

FIG. 6. Conventional PAGE of the soluble HA purified from CA401, demonstrating protease activity associated with the purified protein. We loaded 4 μg of the pI 6.3 purified soluble HA on duplicate non-SDS tube gels. Gels were electrophoresed 90 min at 2 mA per gel constant current. Subsequently, one gel was stained for protein as described in Methods and Materials, whereas the other was embedded in 1.5% skim milk (Difco) agar (20). Specific anti-HA serum was placed in the trough to the right of the embedded gel, and preimmune serum was put in the trough to the left of the gel. After overnight incubation at 25°C, the protein-stained gel was layed on the skim milk plate (partially obscuring the trough containing normal serum). The anti-HA serum inhibited the proteolysis which coincided with the protein-stained band.

Precipitin reactions of identity, without any detectable spurs, were observed between the antiserum and supernatants from CA401, CA411, and 17 grown in either syncase broth or in TSB. Supernatants from these cultures assayed at the time immunodiffusion was performed had significant HA titers (see Fig. 8 legend). Cultures in syncase broth, which is not optimal for HA production, had weaker bands. A hypotoxigenic mutant of 569B, M13, which did not produce detectable HA in this test, also failed to show a precipitin band with the anti-HA antiserum. Strain 3083, an Ogawa serotype El Tor biotype strain, produced an identical soluble HA antigen (results not shown), as did the Texas Star-SR mutant, derived from it (9). The correlation (HA titer-Ouchterlony precipitin band) further supports the hypothesis that the bands observed are HA–anti-HA precipitin bands. Studies using antisera against the three pH isotypes confirmed that they were also immunologically identical (results not shown).

Although, in contrast to previous studies (4), purified HA did not significantly inhibit attachment of *V. cholerae* to infant rabbit tissue (perhaps because of its aggregated state), Fab fragments from specific anti-HA serum markedly inhibited attachment in comparison with Fab fragments from preimmune serum ($P < 0.025$) (Fig. 9). Preimmune Fab fragments were also somewhat inhibitory when compared with the PBS control ($P < 0.025$). A similar observation had been made previously (4). Significant ($P < 0.025$) inhibition of binding by purified HA was demonstrated in the in vivo experiment (results not shown). Similar to previous experience (4), this had a dosage optimum at 10 μg: higher or lower doses were less effective.

DISCUSSION

Of the hemagglutinins produced by cholera vibrios (7), we consider the soluble HA, which was isolated and characterized in this study, to be the most likely to play a significant role in the pathogenesis and immunology of cholera. In contrast to the other HAs, the soluble HA is present in all of the *V. cholerae* cultures tested. In previous work (4), partially purified material isolated from an Inaba serotype *V. cholerae* strain of the classical biotype, was found to inhibit attachment of Ogawa serotype vibrios of the El Tor biotype to infant rabbit ileum in vivo. Presumably, the HA (called cholera lectin in that study) acted by occupying, or otherwise affecting, receptors on the surface of the intestinal epithelium. It was noted that the material was less effective when it was administered simultaneous with, rather than before, the challenge vibrios and it had a dosage optimum. It should also be mentioned that, in contrast to the other

TABLE 3. Effect of temperature on protease and HA activities of soluble HA from *V. cholerae* CA401[a]

Temp (°C)[b]	HA titer	Protease U[c]
4	128	1
25	128	100
37	512	>200

[a] Ammonium sulfate-precipitated crude soluble HA.

[b] All reactants were equilibrated to, and the assay entirely performed at, the designated temperature.

[c] Protease activity was assayed by using the azoalbumin substrate. One protease unit will digest 0.1 mg of azoalbumin in 1 h.

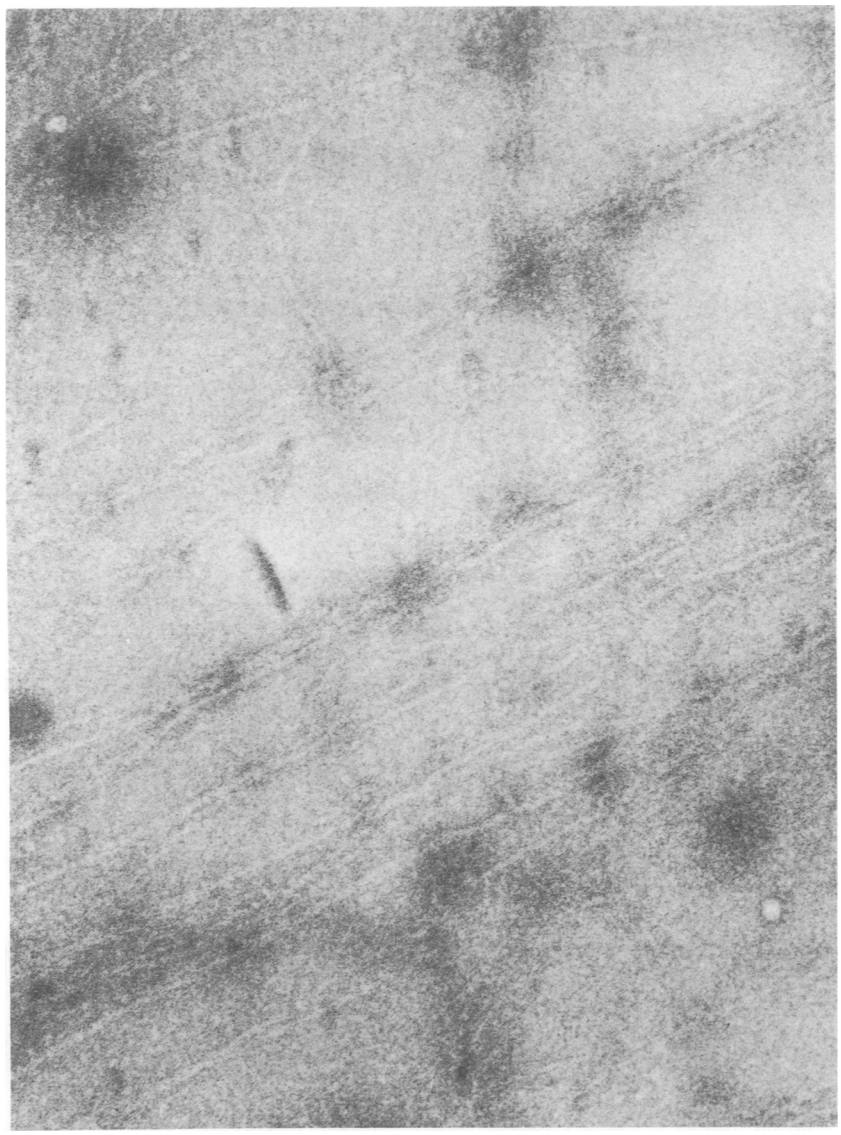

FIG. 7. Electron micrograph of soluble pI 6.3 HA purified from CA401. A total of 500 μg of HA per ml in PBS was spread onto a 200-mesh carbon-coated Formvar grid, then stained with 2% uranyl acetate. Total magnification, ×60,000.

HAs, this soluble HA has a distinguishing characteristic: it is highly active on erythrocytes from some chickens but not others (4, 7).

The question of why cholera vibrios elaborate a soluble (potential) adhesive factor is of major concern. A priori, this would be disadvantageous to the vibrios unless the primary purpose of the factor was to promote detachment (which could be advantageous in enabling the vibrios to be shed and thus find new hosts). Several pieces of evidence, although not conclusive, suggest that the soluble nature of the HA may be a peculiarity of in vitro cultivation. It should be noted that the factor is only found in the supernatant of late log-phase cultures (7) at a time when significant lysis and release of membrane fragments (8) is occurring. Indeed, a significant portion of the activity appears in the large-molecular-size peak in gel filtration (Fig. 1) which contains lipopolysaccharide and other membrane components. That it occurs in a more cell-associated form in vivo is indicated by our observation that washed Inaba serotype classical biotype *V. cholerae*, harvested from the

TABLE 4. Amino acid composition of purified soluble HA from *V. cholerae* CA401[a]

	No. of residues per molecule[b]	% Composition
Aspartate/asparagine	32	14
Threonine	18	7.5
Serine	16	6.2
Glutamate/glutamine	14	6.2
Proline	6	2.7
Glycine	14	6.4
Alanine	12	5.2
Cystine	2	0.96
Valine	14	6.1
Methionine	2	0.87
Isoleucine	6	2.5
Leucine	10	4.3
Tyrosine	16	6.8
Phenylalanine	18	7.9
Histidine	6	2.3
Lysine	8	3.3
Arginine	12	5.1
Tryptophan	6	2.4

[a] Performed by C. W. Gehrke, College of Agriculture, University of Missouri at Columbia, by ion-exchange methodology (1).

[b] Numbers of residues are given as the nearest whole number. Calculations are based on the SDS-PAGE subunit molecular weight determination of 32,000.

intestinal fluids of infected infant rabbits, directly agglutinated responder but not nonresponder, chicken erythrocytes (L. F, Hanne, M. B. Finkelstein, and R. A. Finkelstein, unpublished observations) as well as by the in vivo inhibition of binding studies (4 and the present study). It is also conceivable that the factor could serve the dual function of promoting attachment and detachment.

This study provides convincing evidence that the HA is also a protease. We were led to this observation by the tremendous losses of activity during purification and by its heat lability. The material, isolated to apparent homogeneity after a sequence of gel filtration and IEF, was markedly proteolytic. This protease activity comigrated with the HA activity and was eluted, with the HA, from disc electrophoresis gels run under nondissociating conditions. Both activities were coincident with the single protein-staining band observed in parallel stained gels. Further, the protease activity was inhibited by antiserum, raised against the purified HA, which gives only a single band of precipitation against concentrated crude HA-containing culture supernatants of a variety of *V. cholerae* cultures despite repeated booster doses of the antigen over many months. This band gives a reaction of identity with the purified HA. Fab fragments of this antiserum were also shown to inhibit attachment. Three different pI isotypes of the HA, with pIs of 6.3, 5.3, and 4.7, were detected. Although the latter two were present in lesser amounts and were not completely purified, protease activity was associated with each and they each were immunologically and structurally indistinguishable from the major, pI 6.3, material. The amino acid composition of the pI 6.3 species is provided (Table 4). No carbohydrate or lipid was detected.

Each pI isotype, after isolation, formed a large-molecular-weight oligomer of noncovalently associated subunits of 32,000 as revealed by SDS-PAGE (Fig. 3) performed before and after heating in SDS. That polymerization occurs after isolation is established by the observation that the material selected for purification emerged after the void volume peak from Sephadex G75 (Fig. 1). This probably represents a heterogeneous pool of monomers, dimers, and larger oligomers which have become separated from membrane fragments. The tendency to polymerize could be responsible, at least in part, for the observed loss in HA activity during purification. Electron microscopic observations (Fig. 7) revealed that the isolated material consisted of long filaments or strands. It is also possible that some HA activity is lost by self-digestion due to the associated protease activity. Limited proteolysis could likewise account for the pI isotypes observed. The configuration of the protein in its native state is unknown, but if it is to function as a lectin it should be either di- or

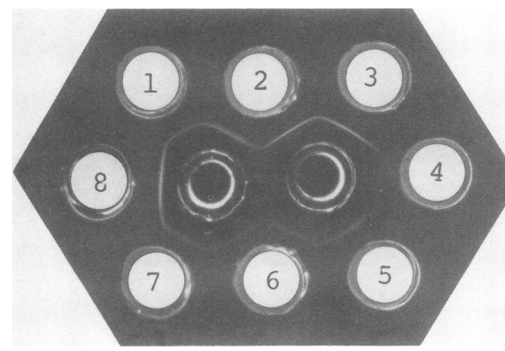

FIG. 8. Immunodiffusion of *V. cholerae* broth cultures against antiserum raised to soluble pI 6.3 HA purified from CA401. Vibrio cultures examined here were grown at 30°C for 20 h on a rotary shaker set at 150 rpm. Soluble HA titers were determined for the cell-free supernatants at the time the immunodiffusion was performed. Center two wells contain antiserum to CA401 IEF pI 6.3. Well 1, strain 17 grown in TSB, titer 256; well 2, CA401 grown in TSB, 256; well 3, CA411 grown in TSB, 256; well 4, M13 grown in TSB, 0; well 5, 17 grown in syncase, 32; well 6, CA401 grown in syncase, 32; well 7, CA411 grown in syncase, 32; and well 8, CA401 crude HA (1:10), 2,048.

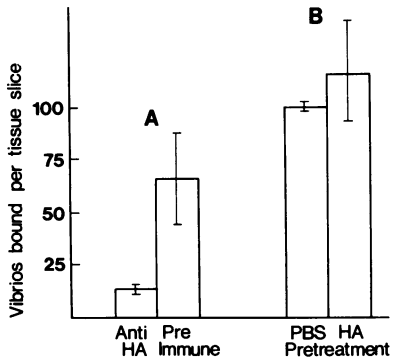

FIG. 9. Effect of soluble pI 6.3 HA purified from *V. cholerae* CA401 or Fab antibody fragments to the HA on attachment of 3083 to infant rabbit ileal tissue. (A) ^{14}C-labeled 3083 was suspended in Fab antibody fragments to the soluble HA in parallel with Fab fragments from preimmune serum as a control. Ileal tissue slices (8 mm) from a 10-day-old infant rabbit were incubated in the vibrio mixture. After 20 min, the slices were removed, rinsed in PBS, placed in scintillation vials, and digested, and the counts per minute were determined. Values are expressed as a percentage of PBS control. Significance: anti-HA Fab versus preimmune Fab, $P < 0.025$; preimmune Fab versus PBS, $P < 0.025$. (B) Tissue sections were pretreated for 15 min at 25°C with either 70 μg of purified soluble HA per ml in PBS or PBS alone. Slices were rinsed and transferred to ^{14}C-labeled 3083 in PBS for 20 min, and then the slices were processed as above. Values are expressed as a percentage of PBS pretreated control. Bars represent the range of values for four tissue slices.

multimeric or anchored in a backbone in the bacterial membrane. Previous information (14, 15) suggests it is not likely to be pilus-like. However, similarly to other microbial adhesins (3, 17, 19, 22, 23), it exhibits hydrophobic properties: it bound rather firmly to the hydrophobic matrix, phenylsepharose (L. F. Hanne and R. A. Finkelstein, unpublished observations), a property which may prove useful in further attempts to purify the material.

Despite the paucity of direct evidence, it is interesting to speculate on the mechanism of action of this HA/protease ratio in vibrio adherence, detachment, or both. Our observations on the temperature requirement of the two activities (Table 3) suggest that they may be independent functions of the same molecule. It is therefore conceivable that one region of the molecule (a hydrophobic domain) is responsible for attachment or that the protease activity prepares the surface for attachment. The protease could likewise potentially function on different substrates at different times and thereby mediate both attachment and, subsequently, detachment. Nelson et al. (14) observed that cholera vibrios apparently do detach during infections in experimental animal models. However, this intriguing phenomenon, although plausible, has not been confirmed in the disease in humans.

It is noteworthy that Woods et al. (24) have recently presented evidence which suggests that trypsin-like proteases from *Pseudomonas aeruginosa* facilitate attachment of these organisms to buccal epithelial cells by their action on host cell-surface fibronectin, a recently characterized class of adhesive, high-molecular-weight glycoproteins (25), which apparently inhibits adherence of these bacteria. It is also relevant to mention that Schneider and Parker (18) have reported that protease-deficient mutants of *V. cholerae* exhibited reduced virulence in infant mice. Antiserum (kindly provided by D. Schneider) against a partially purified *V. cholerae* protease (D. R. Schneider and C. D. Parker, J. Infect. Dis., in press) gave a precipitin reaction of identity with the purified soluble HA and our anti-HA serum (unpublished observations).

From these results and the results of others (6), adherence and colonization by *V. cholerae* is likely to be a multifactorial process involving motility of the vibrios, chemotactic events, and trapping in the mucus gel, the soluble HA-protease-cholera lectin described here, and perhaps the other HAs, neuraminidase, and other factors in certain circumstances. Different factors may be more important in some experimental models than in others, and it remains to be determined which factor, or factors, is the most important in the natural host, the human being. It would be of great interest to ascertain whether immunization with the purified HA, of this work, is protective in animal models and whether antibody to this HA contributes to the immunity which has been observed in humans after convalescence from the disease (11) or after oral immunization with the Texas Star-SR A-B$^+$-attenuated mutant candidate vaccine strain. (M. M. Levine, R. E. Black, M. L. Clements, C. R. Young, T. Honda, and R. A. Finkelstein, Proceedings of the 17th Joint Conference on Cholera, U.S. Japan Cooperative Medical Science Program, in press).

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