Purification and Characterization of 55K, a Major Cytoskeletal Protein from *Vibrio cholerae* O1*

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Abstract: A major cytoskeletal protein of MW 55K was purified from the soluble fraction of French−pressed cells of *Vibrio cholerae* O1 cultured on TCG agar (Ehara *et al.*, 1986) by gel filtration through Sephadex G−100 and ion−exchange chromatography with DEAE−Sephadex A−50. This protein showed a doublet band on SDS−urea−PAGE and isoelectric focusing, although a single band was seen on SDS−PAGE. The amino acid composition of the 55K protein had a molar ratio similar to that of tubulin.

Although the 55K protein was easily extracted from vibrio cells by homogenization in the presence of Mg^{++} and EGTA, it was found to be localized around cell poles and in the cytoplasm.

Taxol was effective in stabilizing the 55K protein around the cell poles and in the cytoplasm. Anti−tubulin antiserum recognized the 55K doublet band of a non−O1 *Vibrio cholerae*, as well as the 55K protein of *Vibrio cholerae*.

The 55K protein was present commonly among the strains of Family *Vibrionaceae*. Tetramers presumably composed of the 55K protein were isolated from the cell surface.

Key words: *Vibrio cholerae*, Cytoskeletal protein, Tubulin

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INTRODUCTION

Vibrio cholerae is a Gram−negative bacterium with a comma−like structure. Little is known about the proteins involved in cell−shape, cell division and secretory function. Iterson *et al.* (1967) reported the presence of filaments about 200A wide similar to microtubules in *Proteus mirabilis*, but the filament has not been analyzed biochemically. The presence of microtubules in prokaryote was reported by Margulis *et al.* (1978) Hargreaves and Avila (1985) have reported the presence of tubulin−like proteins associated with brain mitochondria.

Berk *et al.* (1985) detected actin like material in *Pseudomonas aeruginosa* by a passive transfer immunoblotting, although the protein showed a doublet band with a molecular weight of 60K and 63K which was different from the 43K for actin.

Begg and Donachie (1984) confirmed a polar concentration of *Omp A* protein in *Escherichia coli*, however the transport system of newly synthesized *Omp A* protein to the poles is unclear.

Considering the morphological property of *Vibrio cholerae* O1, its cytoplasm should contain cytoskeletal proteins that change reversibly from monomers to polymers.

In this study, the major cytoskeletal protein in *Vibrio cholerae* O1 was purified and its function within the vibrio cell was examined.

MATERIALS AND METHODS

Strains: The *Vibrio cholerae* strain K23, El Tor biotype, Ogawa serotype isolated from a cholera patient in Kenya in 1984, was grown on TCG agar at 30℃ in Roux bottles. Cells from 5 liters of a 48h culture were harvested by pipetting after the addition of 10ml of F−buffer 1 and centrifuged (15,000×g for 30 min). The pellet (20 g, wet weight) was suspended in cooled F−buffer 1 (0.5 g, wet weight/ml) and the cells were fractured with a French−press (X−Press, AB BIOX, Sweden) The French−pressed sample was centrifuged at 48,000×g for 2 h to remove cell debris and flagella. The supernate was further centrifuged at 190,000×g for 1 h. The resultant supernate was concentrated with Amicon PM10 to 4 ml and the concentrated sample was processed for further purification (S1) . The other strains of Family *Vibrionaceae, i.e.*, non−O1 *Vibrio cholerae, Vibrio mimicus, Aeromonas hydrophila, Plesiomonas shigelloides*, and *Vibrio parahemolyticus* were all clinical isolates in Kenya and Japan.

These strains were also cultured on TCG agar and the soluble fractions obtained by 100,000×g−1 h−centrifugation of French−pressed cells were used in the Western blot analysis.

Temperatures were between 0 and 4℃ unless otherwise specified.

Buffers used: F−buffer 1; 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM MES, pH 6.85, 1 mM ATP, 0.5 mM DTT, 1 mM TAME.

F−buffer 2; 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM MES, pH 6.85, 0.2 mM ATP, 0.5 mM DTT, 1 mM TAME.

PPB; 0.1 M potassium phosphate buffer, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.85.

MES buffer; 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, pH 6.85.

Preparation of the supernatant of homogenized cells: After a 48 h of culture on TCG agar in 50 Roux bottles (100 ml of the medium/bottle) cells were harvested by pipetting after the addition of 10 ml of PPB to each bottle and were centrifuged at 15,000×*g* for 30 min. The pellet (20 g, wet weight) was suspended in the same buffer at the concentration of 0.5 g (wet weight) per 1 ml. The suspension was blended with a Sorvall Omnimixer (DuPont) at grade 5 for 5 min, and then centrifuged at 22,000×*g* for 30 min. The pellet was resuspended in the same buffer at the same concentration. Homogenization was repeated for two more cycles. The supernatants of 3 cycles of homogenization were combined and kept at 4℃ overnight after the addition of DNase I and RNase (Sigma products), 100 μg/ml respectively.

The mixture was centrifuged at 48,000×*g* for 2 h to remove cell debris and flagella and the supernatant was concentrated with Amicon PM10 to 10 ml. The concentrated sample was further centrifuged at 190,000×*g* for 1 h. A small portion of the supernatant was used as the material for SDS−PAGE and EM analysis. The rest was further concentrated with Amicon PM10 to 2 ml for CsCl (1.29 g/cm^3) density gradient centrifugation.

The density gradient centrifugation was performed on a Beckman SW41 rotor at 190,000×*g* for 16 h. Fractionation was done by taking 0.5 ml from the top of the tube and each fraction was checked by EM and SDS−PAGE.

Purification of the major cytoskeletal protein: In general, purification steps that have proven useful for muscle actin (Rees and Young, 1967), Acanthamoeba actin (Gordon *et al.*, 1976) and Dictyostelium actin (Uyemura *et al.*, 1978), sea urchin egg actin (Mabuchi and Spudich, 1980) proved useful for a major cytoskeletal protein of *Vibrio cholerae* as well. S1 was applied to a Sephadex G−100 column (2.5×45 cm) that had been equilibrated with F−buffer 2. As described in the "Results" section, those fractions showing a major band in SDS−PAGE were selected as containing a major cytoskeletal protein. Therefore, this experiment was focused on the relationship between a major band and a major cytoskeletal protein. Fractions 22 to 27 (30 ml) eluted after the void volume fraction were combined and concentrated with Amicon PM10 to 4 ml and then passed through a DEAE−Sephadex A−50 column (0.9×30 cm), which had been washed with F−buffer 2. After application of the sample, the column was washed with 100 ml of F−buffer 2 and then proteins were eluted with 400 ml of a linear gradient of 0.1−0.5 M KCl.

Analytical methods: The protein concentration was measured by the procedure of Lowry *et al.* (1951), using bovine serum albumin as a standard.

SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed by the method of Laemmli (1970) using 9% and 12% polyacrylamide gels. Molecular weights were estimated by coelectrophoresis of major cytoskeletal protein samples with standard proteins (LMW−electrophoresis calibration kit from Pharmacia). For SDS−urea−PAGE,

samples were treated with sampling buffer containing 8 M urea. Amino acid analysis was carried out on the major cytoskeletal protein eluted from a preparative SDS−PAGE.

The extracted major cytoskeletal protein solution was first dialyzed against 0.2 M NaCl to remove glycine in the electrophoresis buffer before dialysis against distilled water.

The samples were hydrolyzed with 6 N HCl at 110℃ for 24 h in evacuated sealed tubes.

The hydrolysates were analyzed with a JEOL JLC−200A amino acid analyzer. Isoelectric focusing of the major cytoskeletal protein was carried out with an Ampholine PAG PLATE (LKB) at pH 3.5−9.5.

Electron microscopy: Samples were mounted on a carbon−coated formvar grid, negatively stained with 1% aqueous uranyl acetate and viewed with a JEOL−100CX electron microscope.

Preparation of antisera: Porcine brain tubulin purified through 3 cycles of polymerization and depolymerization (Shelanski *et al.*, 1970) was run onto a preparative SDS−PAGE to remove microtubule−associated proteins (MAPs). The tubulin band was cut off the gel and electrophoretically extracted. DEAE−purified 55K protein of *Vibrio cholerae* was also further purified by a preparative SDS−PAGE to remove minor protein contaminations.

Each antigen was injected to different rabbits after mixing with an equal volume of complete Freund adjuvant (0.5 ml of the protein solution, 200 μg/ml, respectively and 0.5 ml of the adjuvant).

Injections were performed subcutaneously at two sites of the back and intramuscularly at two sites of the thigh. The booster injections, which contained incomplete Freund adjuvant in place of complete Freund adjuvant were carried out 4 times every two weeks.

One week after the last injection, rabbits were bled from a carotid artery. IgG fractions were obtained by a DEAE−Sephadex A−50 column chromatography after precipitating immunoglobulin with 33.3% ammonium sulfate. Each antisera was adjusted to a concentration of 6 mg/ml after overnight dialysis in phosphate buffered saline (pH 7.2) and lyophilized.

Western blot procedure: Western blot analysis was performed according to the method of Burnett (1982). Samples from various origins were electroblotted from 9% SDS−polyacrylamide gels (1.5 mm) onto nitro−cellulose sheets (pore size, 0.45 μm; BIO−RAD) for 16 h at 30 V and for 1 h at 70 V and 4℃. The anti−tubulin antibody was used at a concentration of 250 μg/ml and reacted overnight at 4℃. Anti−55K protein antibody was used after a 400−fold dilution of the lyophilized antiserum and reacted for 90 min with shaking. Peroxidase−labeled goat anti−rabbit IgG (Cappel Laboratories, West Chester, Pa.) was used to detect rabbit IgG after a 400−fold dilution. Controls consisted of rabbit preimmune serum.

Localization of a major cytoskeletal protein: *Vibrio cholerae*, strain of K23

cultured on TCG agar overnight at 37℃ were transfered into TCG broth (3×10^8 CFU/ml) and further incubated at 37℃ for 3 h in the presence or absence of 25 μM taxol solubilized in DMSO. Taxol was a kind gift from Dr. M. Suffness, Natural products Branch, Division of Cancer Treatment, National Institute of Health, Bethesda, Maryland 20205, U.S.A.

The concentration of DMSO in the medium was kept lower than 1%. Cells were harvested by centrifugation at $10,000\times g$ for 30 min at 25℃. Cell pellets were fixed with 2 % glutaraldehyde in MES buffer at 25℃ for 1 h and then washed 3 times.

Cell washing was performed with MES buffer by centrifugation at $10,000\times g$ for 15 min. Cells were treated in 90% acetone with 20 mM EGTA, pH 6.8 at −20℃ for 10 min and washed 3 times. Samples were coated with 1% BSA in MES buffer for 30 min at 37 ℃, and then washed twice. Each sample was reacted with anti−55K protein antiserum solution (5 mg IgG/ml) for 30 min at 37℃.

Five nm−colloidal gold labeled anti−rabbit IgG goat serum (E.Y. Laboratories) was used to detect rabbit IgG. The colloidal gold conjugated antibody gives pink coloration of antigenic sites under a light microscope.

Results

Purification of the major cytoskeletal protein

The major cytoskeletal protein fractions eluted after the void volume fraction (Fig. 1) were combined and passed through a DEAE−Sephadex A−50 column chromatography. A major cytoskeletal protein was eluted at 0.35 M KCl showing a single band on SDS−PAGE, although a doublet band was shown on SDS−urea−PAGE.

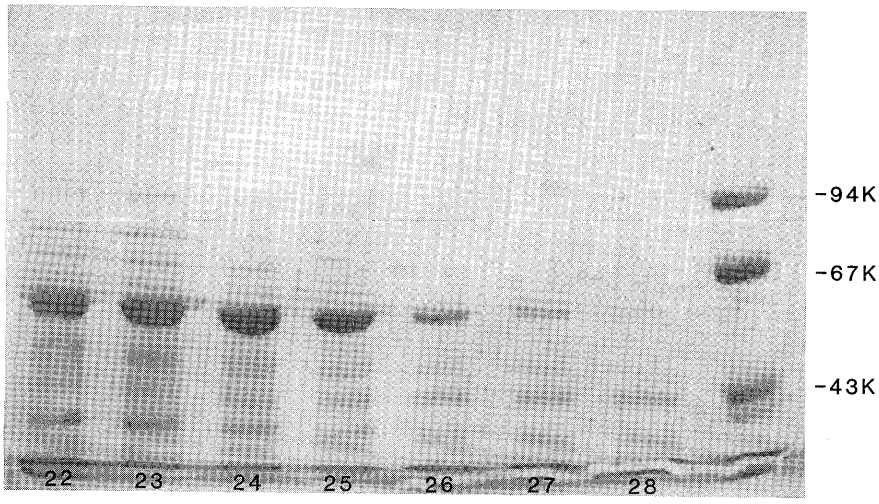

Fig. 1. SDS−PAGE (9% gel) pattern of the eluted fractions. Marker proteins are rabbit muscle phosphorylase b(MW. 94,000), bovine serum albumin(MW. 67,000), and egg white ovalbumin(MW. 43,000)

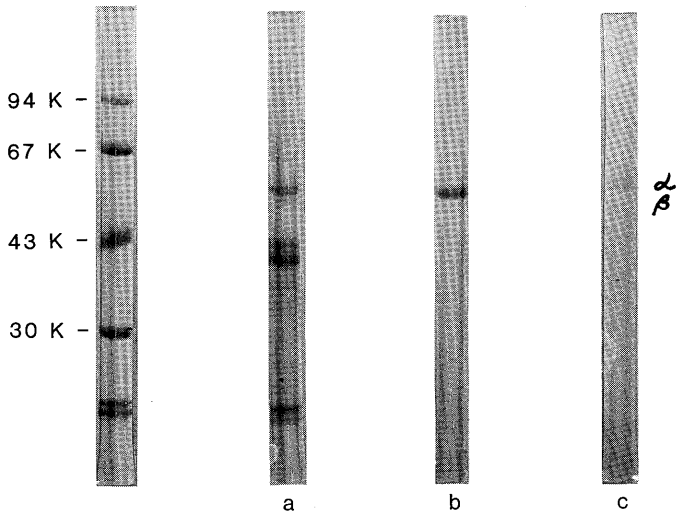

Fig. 2. Gel electrophoretic patterns of crude and purified 55K protein from *V. cholerae* O1 a and b, SDS−PAGE (Laemmli); C, SDS−Urea−PAGE.
a, supernatant of 190,000×g−2 h−centrifugation of homogenized cells cultured on TCG agar.
b, DEAE−purified 55K protein.
c, same sample in b.

The molecular weight of a major cytoskeletal protein was about 55K (Fig. 2). Fig. 3 shows an isoelectric focusing of the DEAE−purified 55K protein. Two major bands were identified between pI 5.20 to 5.85 with several isomers.

Amino acid composition

A quantitative amino acid analysis was performed on the 55K protein purified by a preparative SDS−PAGE (Tab. 1). The amino acid composition was characterized by high concentrations of glycine, glutamic acid and aspartic acid. Values of Ser and Gly residues may be over estimated due to contamination during the preparative SDS−PAGE.

Isolation of tetramers from vibrio cell−surface

The supernatant of the 190,000×*g*−1 h−centrifugation of homogenized cells was processed for EM and SDS−PAGE before

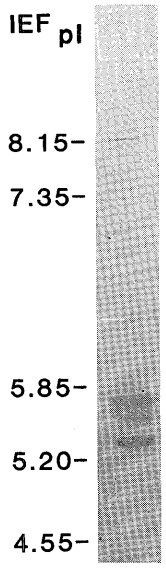

Fig. 3. Isoelectric focusing of DEAE−purified 55K protein from *V. cholerae* O1.

Table. 1 Amino acid composition of 55K protein of *Vibrio cholerae*

Amino acid	Residue/molecule	Assumed value
Asx	62.06	62
Thr	32.90	33
Ser	39.77	40
Glx	67.67	68
Pro	21.81	22
Gly	74.98	75
Ala	29.86	30
Val	26.31	27
Met	4.75	5
Ile	17.43	18
Leu	34.73	35
Tyr	10.87	11
Phe	13.08	13
His	14.54	15
Lys	36.20	37
Arg	13.80	14
Trp	N.D.	N.D.

N.D., not determined.

CsCl density gradient centrifugation. Fig. 4 shows numerous tetramers of a pair of dumbbell structures together with fimbriae. The main band of the sample on SDS−PAGE was also the 55K protein. Fimbrilin of *Vibrio cholerae* is a 16K protein (unpublished data). The homogenized cells were mostly intact under EM, although they lacked surface appendages.

CsCl density gradient centrifugation

After the centrifugation, the sample was fractionated by carefully removing 0.5 ml of the solution from the top of the tube. Each fraction was checked by EM and SDS−PAGE. As shown in Fig. 5, the 55K protein was seen from the middle to the bottom fraction showing amorphous aggregates under EM (Data not shown). These fractions suggested that the 55K protein forms several sizes of polymers in the cytosol of vibrio cells, which are easily inactivated under the present experimental conditions.

Western blot

The anti−tubulin antibody recognized the 55K protein as shown in Fig. 6, however the anti−55K protein antibody did not recognize porcine tubulin (Fig. 7) . The anti−tubulin antibody recognized the doublet band like α−and β−tubulin in the soluble fraction of French−pressed non−O1 *Vibrio cholerae* together with 2 minor bands near 60 K (Fig. 8) . The anti−55K protein antibody recognized the 55K proteins commonly present among the strains of the Family *Vibrionaceae*. The immunostain patterns of *Vibrio cholerae* O1, non−O1 *Vibrio cholerae* and *Vibrio mimicus* were quite similar. The 55K pro-

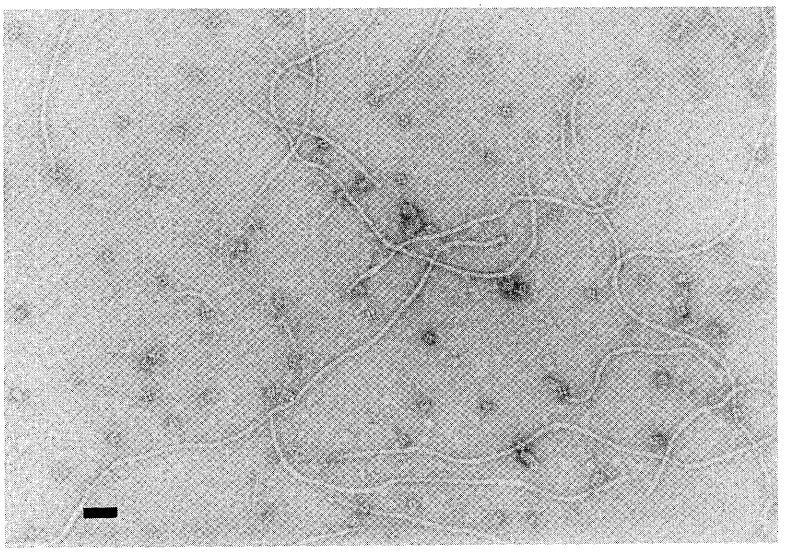

Fig. 4. Negatively stained supernatant of 190,000×g−2 h−centrifugation of homogenized cells of strain K23 showing numerous tetramers and fimbriae.
Bar indicates 50 nm.

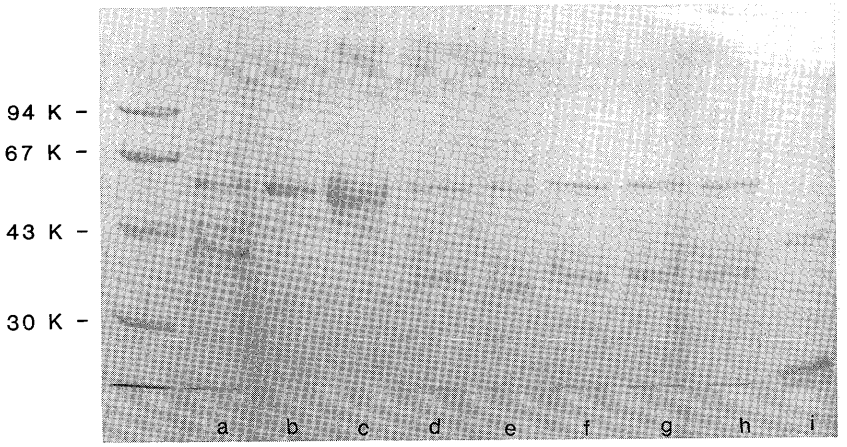

Fig. 5. Gel electrophoretic patterns of tubulin and 55K protein (Laemmli, 9% gel). a, supernatant of 190,000×g−2 h−centrifugation of homogenized cells cultured on TCG agar. b, DEAE−purified 55K protein from *V. cholerae* O1. c, porcine brain tubulin purified by 3 cycles of polymerization and depolymerization d−h, fractions No.20, 18, 16, 14, 12 of CsCl density gradient centrifugation (1.29 g/cm³). i, purified cholera toxin.

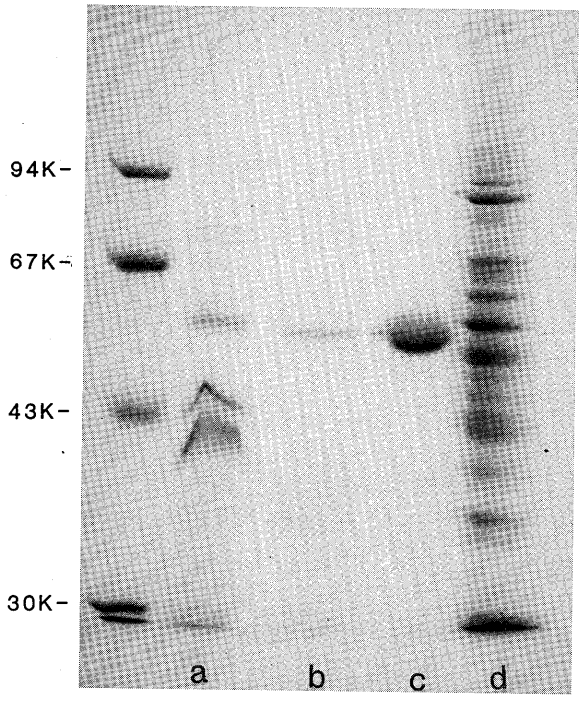

Fig. 6a. SDS−PAGE (9% gel) pattern.
a) supernatant of 190,000×g−2 h−centrifugation of homogenized cells cultured on TCG agar.
b) fraction No.12 of CsCl density gradient centrifugation (1.29 g/cm^3).
c) DEAE−purified 55K protein from *V. cholerae* O1.
d) supernatant of 190,000×g−2 h−centrifugation of homogenized rat brain.

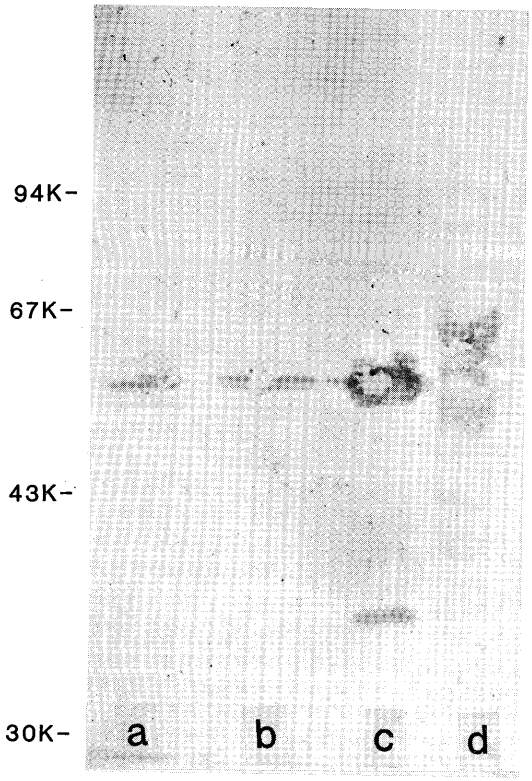

Fig. 6b. Western blot profile of tubulin and 55 K protein from different sources against antiserum raised to porcine brain tubulin.

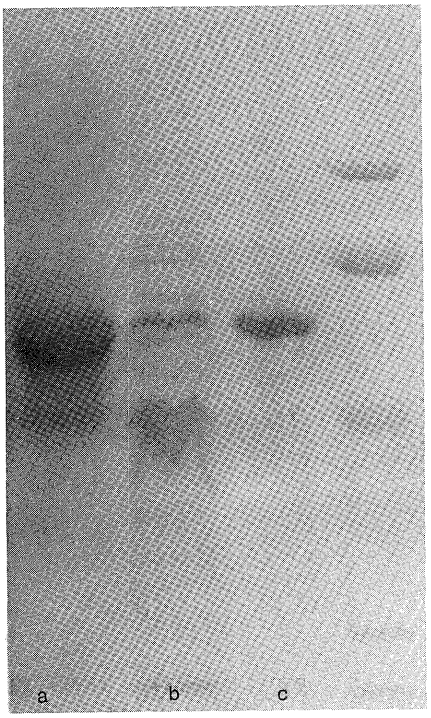

Fig. 7a. SDS−gel electrophoretic patterns (9% Laemmli).
a. porcine brain tubulin purified by 3 cycles of polymerization and depolymerization.
b. supernatant of 190,000×g−2 h−centrifugation of homogenized cells.
c. DEAE−purified 55K protein.

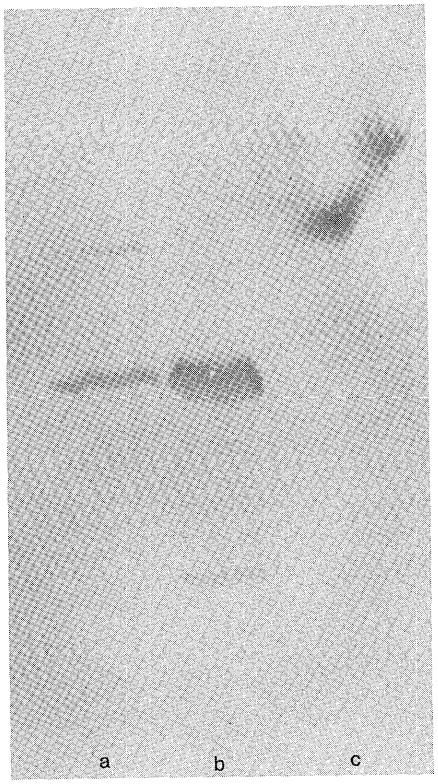

Fig. 7b. Western blot of 55K protein and tubulin
a. supernatant of 190,000×g−2 h−centrifugation of homogenized cells cultured on TCG agar.
b. DEAE−purified 55K protein.
c. porcine brain tubulin purified by 3 cycles of polymerization and depolymerization

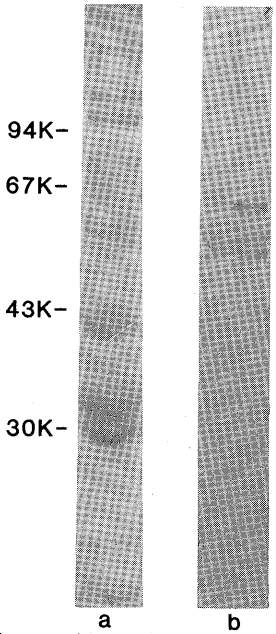

Fig. 8. Immunoblot of 100,000×g−supernate of non−O1 *V. cholerae* against antiserum raised to porcine brain tubulin.
a) SDS−urea−PAGE (Laemmli, 9 %gel, 8 M urea)
b) Immunoblot

tein in these 3 species was shown to be a major soluble protein (Fig. 9). In some experiments, the anti−55K antibody recognized a doublet band near 55K without the treatment of the sample with 8 M urea (Fig. 10).

Preimmune rabbit sera did not recognize any specific band in any immunostain (Data not shown).

Localization of the 55K protein in vibrio cells

Every vibrio cell was stained a wine color with colloidal gold particles. The 55K protein was localized around cell poles more clearly in the cells treated with taxol compared to the non−taxol−treated cells. Control cells reacted with rabbit preimmune serum did not have any coloration from the inside of the cells (Fig. 11). No immunostain was observed around the surface of the cells in any samples tested.

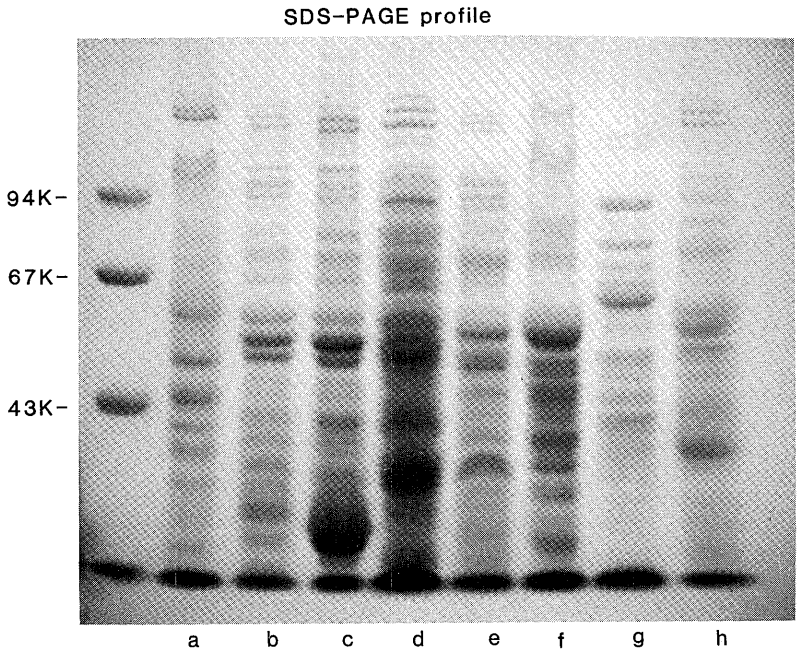

Fig. 9a. 100,000×g−supernate of French pressed cells of Vibrionaceae and their relative.
a) *Plesiomonas shigelloides* b) *V. cholerae* O1 (82P6)
c) Non−O1 *V. cholerae* d) *V. parahemolyticus*
e) *V. mimicus* f) *Aeromonas hydrophila*
g) *Campylobacter jejuni* h) *V. parahemolyticus*

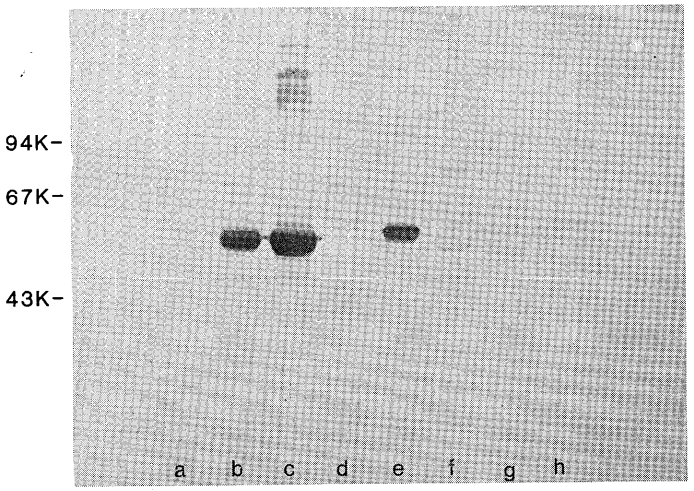

Fig. 9b. Immunoblot of 100,000×g−supernate of Vibrionaceae and their relative against antiserum raised to 55K protein of *V. cholerae* O1.

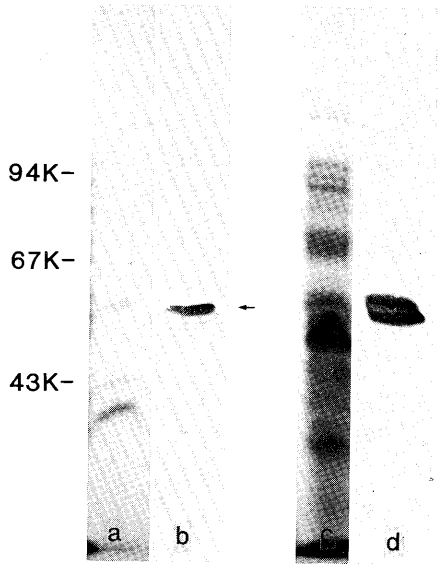

Fig. 10. Western blot profile of 55K protein against anti−55K protein antibody showing β−configuration (arrows).
lanes a,c: SDS−PAGE,
b,d: western blot.
lanes a,b: fraction No.12 of CsCl density gradient centrifugation, c,d : supernatant of 100,000×g−1 h−centrifugation of French pressed cells of *Vibrio mimicus*.

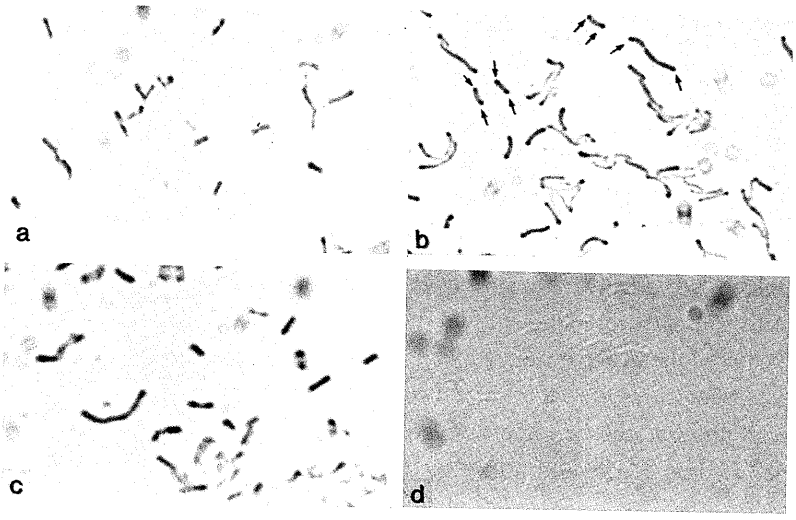

Fig. 11. Localization of the 55K protein in vibrio cells
a) taxol−non−treated cells
b,c) cells treated with taxol with different focusing
d) control cells

Arrows indicate cell−poles.

DISCUSSION

The role of contractile protein in bacteria is not clear.

Since Neinmark (1977) reported the isolation of an actin−like protein (MW 45,000) from the prokaryote *Mycoplasma pneumoniae*, the hypothesis that *Mycoplasmas* contain contractile proteins closely resembling those of eucaryotic cells has attracted much interest.

Meng and Pfister (1980) observed the presence of intracellular thin fibers with a diameter analogous to that of rabbit muscle F−actin. It has recently been shown that group A *streptococci* contain antigens that cross−react with myosin (Krisher and Cunningham, 1985) . In addition, Dale and Beachey (1985) presented evidence that M Proteins from three different serotypes of group A *streptococci* share epitopes with cardiac myosin, but not α−tropomyosin, actin or myosin light chains. Townsend *et al.* (1980) purified fibers 3.5 nm in diameter from the honeybee spiroplasma (BC3). The protein was composed of a single protein which had a MW of 55,000. The MW of the monomer is similar to that of the tubulin subunit (MW 55,000), which occurs in all eukaryotic cells and polymerizes to produce the 5 nm diameter protofilaments from which microtubules are formed.

Townsend (1983) also observed the presence of a twisted flat ribbon of fibrils in the membrane ghost (BC3), and proposed that fibrils are responsible for maintenance of helical cell shape and rotary motility. He also suggested the role of the fibrils in the segregation of chromosomes during cell division.

The present study shows that the 55K protein of *Vibrio cholerae* is a major cytoskeletal protein with epitopes that reacted with anti−porcine brain tubulin antibody.

The anti−55K protein antibody strongly reacted with the 55K proteins of *Vibrio mimicus* and non−O1 *Vibrio cholerae*, although the antibody weakly reacted with those of *Vibrio parahemolyticus, Plesiomonas shigelloides*, and *Aeromonas hydrophila*.

The anti−55K protein antibody did not react with porcine brain tubulin. Further studies are required before we can explain this controversial finding. The 55K proteins have diverse antigenicities through their own evolutional processes.

We could observe the tetramer structure in the supernatant of homogenized cells. The presence of the tetrameric form of tubulin was reported in the initial step of microtubule formation (Dentrich *et al.*, 1982; Foster and Rosemeyer, 1986) .

Therefore, the tetramers of *Vibrio cholerae* may form microtubules under certain physical buffer conditions. We did not observe the 55K protein in the surface of *Vibrio cholerae*, but the protein was easily extracted from the membrane in the presence of EGTA and Mg^{++} with homogenization. Presumably, part of the 55K protein may be present on the outer membrane as a monomeric form and easily detached during the process of the fixation in the presence of a high concentration of EGTA. We speculate that the interaction between the 55K proteins on the outer membrane and those in the cytoplasm through the inner membrane and the peptidoglycan layer is important in the transport

system and the maintenance of cell shape. None of the many reports on the outer membrane protein of *Vibrio cholerae*, has shown the presence of the 55K protein and the tetrameric structure (Kabir, 1980; 1983a; 1983b; Kelley and Parker,1981; Sciortino and Finkelstein, 1983; Richardson and Parker, 1985; Sciortino *et al.*, 1985) .

In conclusion, the 55K protein of *Vibrio cholerae* is similar to tubulin in the following points; 1) SDS−PAGE profile, SDS−urea−PAGE profile, *i.e.*, MW 55K and a doublet band, 2) isoelectric focusing profile showing several isomers, 3) amino acid composition characterization by a high concentration of acidic amino acid, 4) localization of the protein around the poles and the distribution of the protein in the cytoplasm, 5) common antigenicity with porcine brain tubulin, 6) presence of tetramers with a dumbbell structure, 7) effect of taxol on the 55K protein in the cytoplasm as well as around the poles, 8) conservative nature of the protein as found among the strains of Family *vibrionaceae.*

The 55K protein may be useful in studying the even more fascinating subject of the physiological mechanism of prokaryotic cell division.

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コレラ菌の 55K，主要細胞骨格蛋白の精製及びその性状

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コレラ菌をTCG寒天培地で培養し,そのフレンチ・プレス破砕後の可溶性画分からセファデックスG−100 及び DEAE−セファデックスA−50 を用いて，分子量55,000の主要細胞骨格蛋白を精製した.この蛋白はSDS−PAGE上では単一バンドであるが，SDS−urea−PAGE及び等電点電気泳動では2本のバンドを示した．この蛋白のアミノ酸組成はチューブリンのそれと類似していた．55K蛋白は，Mg^{++}，EGTAの存在下で容易にホモジナイザーで抽出されるが，細胞内の極及び細胞質に存在している事が証明された。Taxolは55K蛋白の極及び細胞質への局在を安定化するのに有効であった．抗チューブリン抗体はコレラ菌及びNon−O1 コレラ菌の 55K蛋白を認識した．この55K蛋白はコレラ菌のみならず，ビブリオ科の株に共通に認められた．55K蛋白より構成されていると思われる4量体が，コレラ菌表層より単離された．

以上の事から，コレラ菌55K蛋白は，真核細胞の細胞骨格蛋白のチューブリンと近縁の蛋白と思われ，原核細胞の分裂機構を解明する上で有効な構造蛋白と思われる．

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