

Purification of Fimbrial Subunits of *Vibrio cholerae* O1 and Its N-terminal Amino Acid Sequences

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Abstract: Fimbrial subunit of *Vibrio cholerae* O1 was purified by affinity chromatography using Cellulofine sulfate. Fimbrial subunit purified by this method showed different sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile depending on the sample sources. Fimbrial subunit showed single band on SDS-PAGE, although it revealed numerous bands on isoelectrofocusing (IEF) suggesting the interaction of fimbriae with lipo-polysaccharide. The molecular weight of the fimbrial subunit was 12 kDa smaller than that reported earlier (Ehara *et al.*, 1987).

Key words: Fimbriae, *Vibrio cholerae* O1.

INTRODUCTION

Vibrio cholerae O1 causes cholera by colonizing the surface of the small intestine of infected patients. The process of colonization appears to be essential to pathogenesis, since strains which are unable to colonize the gut are also unable to cause the disease (Levine *et al.*, 1983). Although it has been suggested that *V. cholerae* haemagglutinins are involved in the adhesion of the bacteria to the brush borders of the intestinal epithelium (Jones *et al.*, 1976), we confirmed the presence of the long flexible fibers on the vibrios scraped from the surface of epithelial cells in a rabbit ileal loop test (Ehara *et al.*, 1986). These long flexible fibers were reproducible on a TCG agar plate, purified and shown to possess haemagglutination activity as reported earlier (Ehara *et al.*, 1987). Here we provide data from affinity chromatography using Cellulofine Sulfate resin (CS resin) to purify fimbrial antigens of *V. cholerae* and the N-terminal amino acid sequence of the fimbrial subunit.

MATERIALS AND METHODS

Bacterial strain and culture condition: Strain K23-7 (El Tor bio-type, Ogawa serotype, non-motile mutant) were cultured on TCG agar in 100 Roux bottles containing 100ml of the medium at 30°C for 48h.

Preparation of the samples for affinity chromatography: After this time, 10ml of TBS (20mM Tris-HCl buffered saline, pH 8.0) was added to each bottle, and vibrio cells were harvested by pipetting. The cell suspension was centrifuged for 1h. All centrifugations were done at 12,000g and at 4°C. The supernate was acidified with 50% trichloroacetic acid (TCA) to a final concentration of 3% TCA, and kept at room temperature for 10 min. The resultant precipitate after centrifugation for 20 min was solubilized with 1M Tris adjusting pH at 8.0 and designated as cell-wash. The cell pellet (120g, wet weight) was suspended in TBS at a concentration of 1 g/ml and followed by homogenization in a Sorvall Omnimixer at grade 6 for 15 min. The homogenization was repeated 3 times successively with 5 min-cooling intervals. The homogenate was centrifuged for 1h and the supernate was designated as shear fraction. The resultant pellet of sheared cells was also acidified with 50% TCA to a final concentration of 3% TCA to kill live vibrios, and after mixing well, was kept at room temperature for 10 min, then centrifuged for 20 min. The pellet was solubilized with 1 M Tris adjusting pH at 8.0 and was further treated with 8 M urea in TBS at 37°C for 4 h followed by centrifugation for 1h. The resultant supernate was designated as urea-extract of sheared whole cells. Cell-wash and shear fraction were also treated with 8M urea to decrease the intrinsic viscosity of the fimbrial preparations. The three samples (cell wash, shear fraction and urea extract of sheared cells) were loaded on to affinity columns together with urea, omitting dialysis in TBS.

Cellulofine sulfate affinity chromatography: Before application of the sample, the gel was washed with 2 M NaCl (10 times of gel bed volume) followed by washing with deionized water and by equilibration with TBS. Each column was washed with 500ml of TBS after sample application. When the eluate showed below 0.005 O.D. at 280 nm of wave length, the elution was started. This technique used for the purification of filamentous haemagglutinin of *Bordetella pertussis* was adapted for the purification of fimbrial subunit of *V. cholerae* through personal communication with Aihara, K, The Chemo-Sero Therapeutic Research Institute, Kumamoto, Japan.

HPLC: Samples for HPLC were dialyzed against distilled water and lyophilized. The lyophilized samples of shear fraction and ureaextract of sheared whole cells were dissolved in 1 ml of 6 M guanidine-HCl solution respectively, then incubated at 37°C for 1h. Column: μ BONDAsphere 5 μ C₈-300A, 3.9mm \times 15cm. Flow rate: 1ml/min. Eluent A: H₂O/0.05% trifluoro acetic acid (TFA). Eluent B: CH₃CN/0.05% TFA. Gradient: 0-80% B, 60min, linear gradient. Detection: UV 220nm.

SDS-PAGE and western blot: Samples were electrophoresed at 15mA per mini-slab for 1h by the method of Laemmli (1970), then stained for protein with Coomassie Brilliant Blue R250. Immunoblotting was performed according to the techniques of Towbin (1979).

Iso-electrofocusing: For IEF, samples were loaded onto a Ampholine PAG PLATE (LKB) after treatment with 6M urea.

N-terminal amino acid sequence of the fimbrial subunit: Samples were run on a protein sequencer system of Applied Biosystem, ABI 477A/120A.

Haemagglutination test: Techniques for the quantification of haemagglutinin activity (HA) was adapted from Jones and Freter (1976).

Electron microscopy: Each specimen was spread onto a 200-mesh carbon coated Formvar grid, then stained with 4% uranyl acetate for 30sec and examined with a JEM 100CX electron microscope operated at 80kV.

RESULTS

Purification of fimbrial subunit by affinity chromatography: The combined elution profiles are shown in Fig. 1a. The yield of urea-extract of sheared whole cells was higher than the yields of the shear fraction and cell-wash samples. Each peak fraction was analysed by SDS-PAGE and electron microscopy (EM).

The haemagglutination (HA) activity of each peak fraction was also examined after dialysis in TBS. Samples taken from the peak fractions of the cell-wash and shear fraction showed HA activity to human type O red blood cells, although resistant to D-mannose as well as L-fucose (data not shown), and were similar to dissociated fimbriae of *V. cholerae* O1 under EM (Fig. 1b). However the sample taken from the peak fraction of urea-extract of sheared whole cells showed no HA activity and showed only oligomeric protein compounds under EM (Fig. 1c). The results of HA activity in all samples correspond to the structures observed under EM. That is, fimbriae show HA properties even if they are broken into short fibrils, but not if they exist in a dissociated form. Figure 2a shows the SDS-PAGE profile of the peak elution fractions of shear fraction. The 80 kDa protein pro-

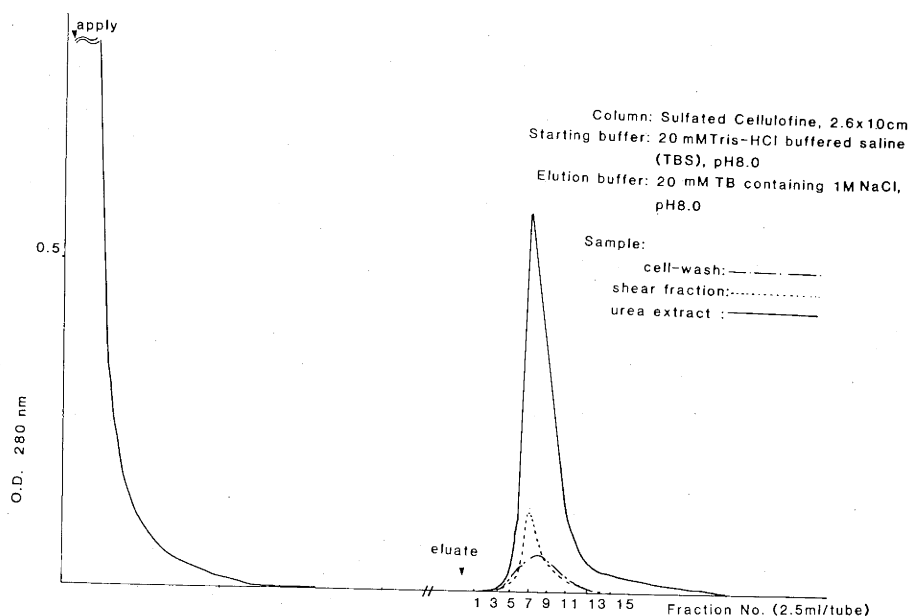


Fig. 1a. Elution profiles of fimbrial preparations from CS resin affinity chromatography.

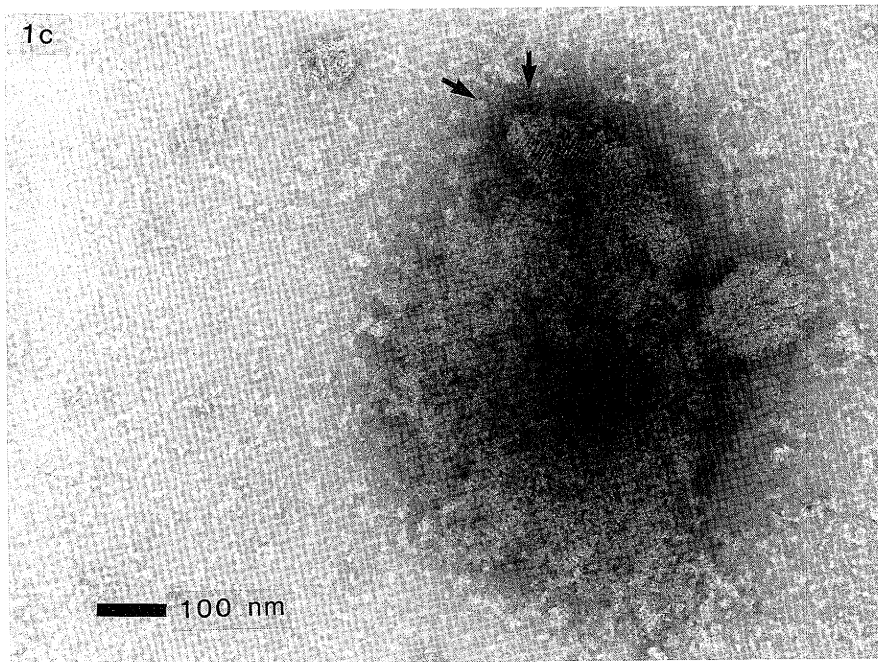
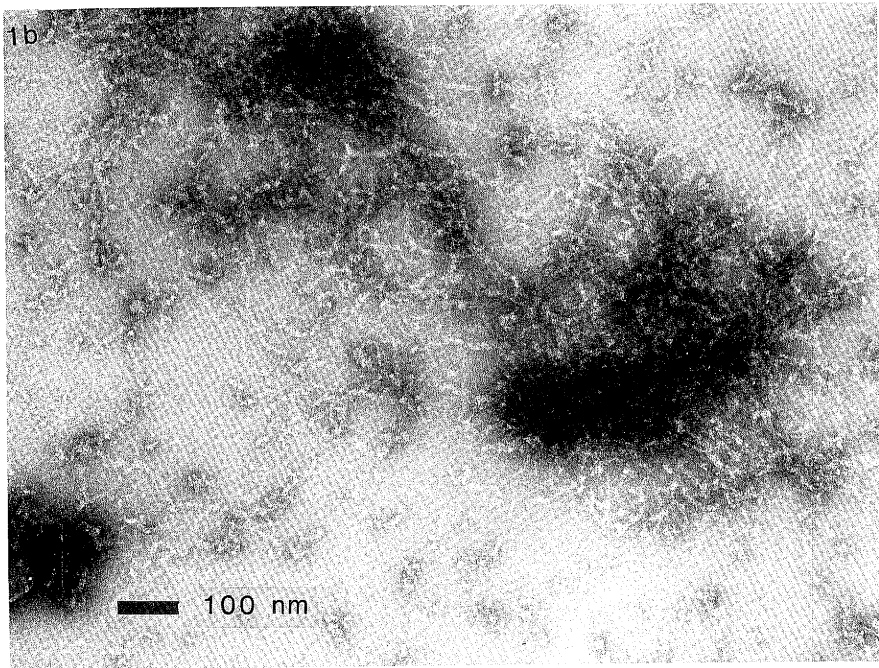


Fig. 1b. and 1c. Electron micrographs of the peak fraction (No. 7).
1b: shear fraction, 1c: urea-extract of sheared whole cells.
Note the paracrystals indicated with arrows.

vides the major band in the lanes for fractions No. 7 to 11. The 80 kDa protein was recognized by both anti-native fimbriae antiserum and anti-electrophoretically purified 16 kDa protein antiserum (Ehara *et al.*, 1987) (Fig. 2b), which suggests that the 80 kDa protein is a homopolymer of the 16 kDa fimbrial subunit. Therefore, we tried to dissociate 80 kDa protein into a doublet band as reported earlier (Ehara *et al.*, 1987). In spite of reducing condition, the 80 kDa protein was not dissociated into smaller fragments as shown in Fig. 2c.

Fractions eluted by affinity column chromatography were combined and loaded on a reversed phase column and chromatographed after treatment with 6M guanidine-HCl. The chromatogram is shown in Fig. 3a and the SDS-PAGE profile of the peak fractions is shown in Fig. 4a. Each fraction yielded the previously observed doublet bands. From the data (Fig. 2 and Fig. 4a), it is clear that 80 kDa protein is a multimer of fimbrial subunits.

Samples taken from fractions 35 and 38 of shear fraction were loaded onto a protein sequencer to determine N-terminal amino acid sequence, the results of which are shown in Fig. 5a. As expected, both fractions showed the same sequences up to 20th amino acid residue from the N-terminal amino acid residue. Identical SDS-PAGE and protein sequence data were obtained from corresponding elution fractions of the cell wash. In the case of urea-extract of sheared cells, the SDS-PAGE profile of the peak fractions eluted from CS resin differed slightly from that of the shear fraction, in that the major band shifted up at ca. MW 100 kDa (data not shown). Each fraction was combined again and reversed phase

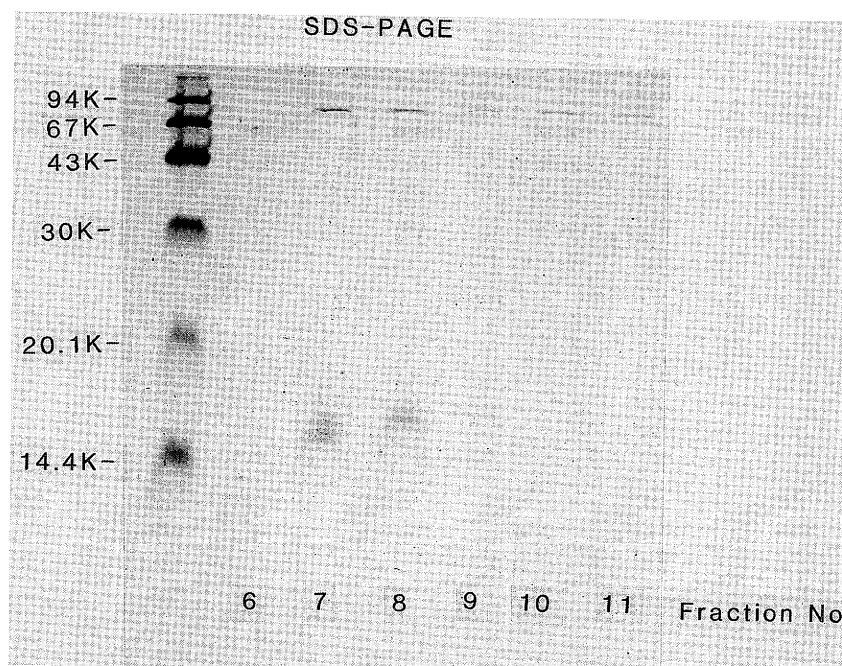


Fig. 2a. SDS-PAGE profile of the shear fraction eluted from resin.

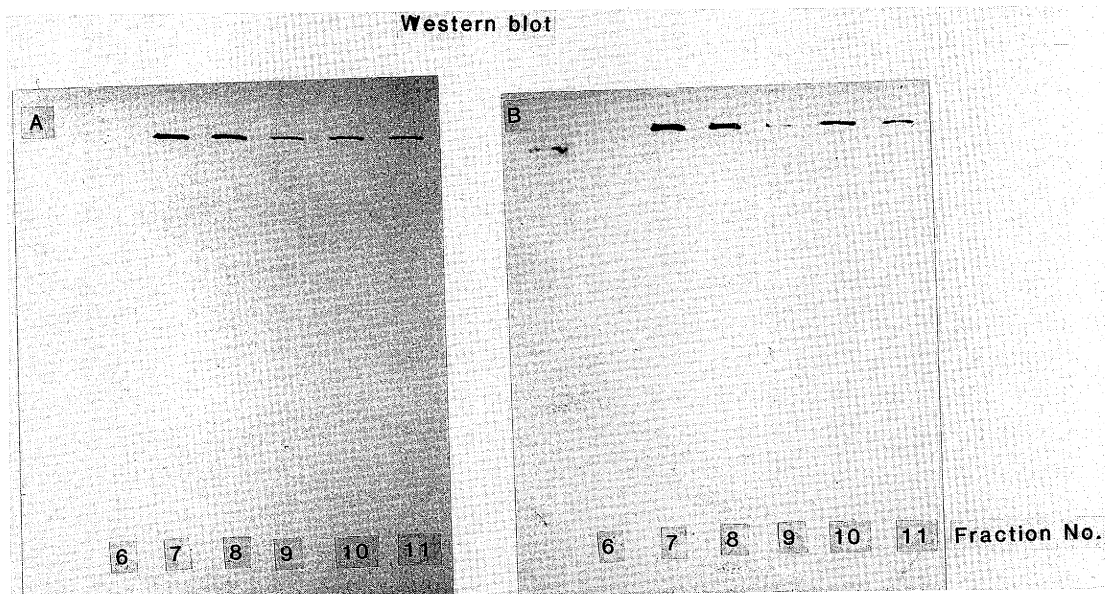


Fig. 2b. Western blot analysis of the shear fraction eluted from CS resin.
 A: reacted with anti-crude fimbriae antiserum.
 B: reacted with anti-16K protein antiserum.

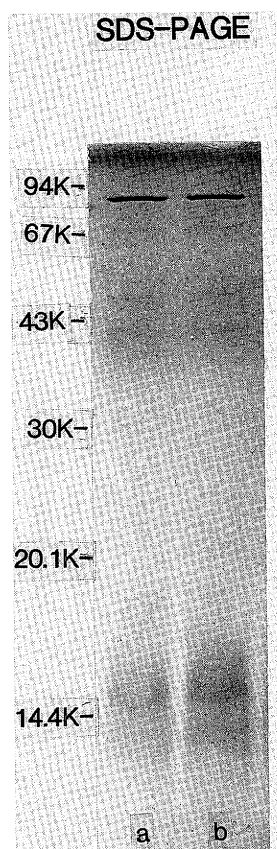


Fig. 2c. Sample: fraction No. 7.
 a: non reduced
 b: reduced with 50 mM DTT in the presence of nitrogen gas

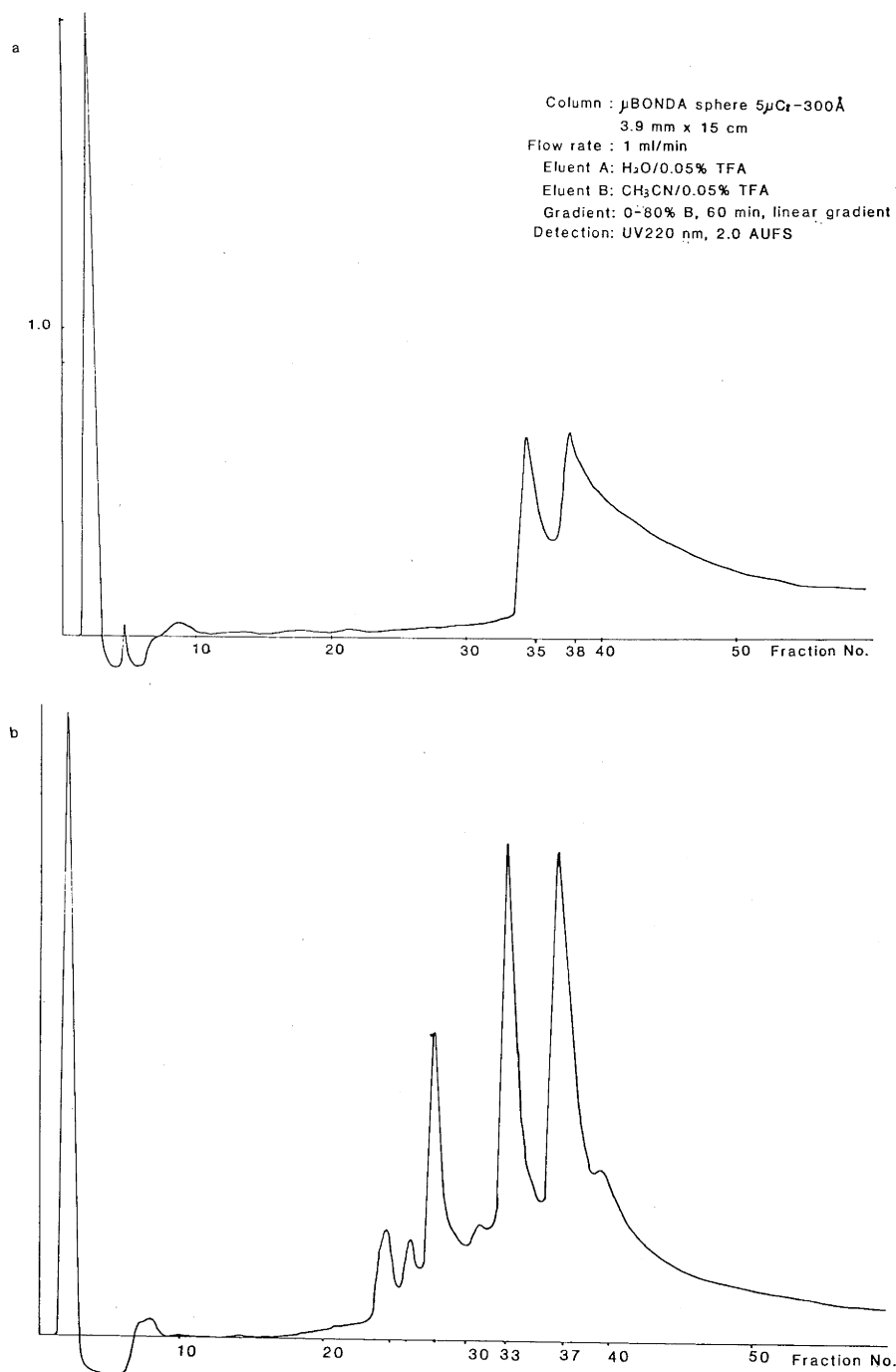


Fig. 3. Elution profile of reversed phase column chromatography of
a: shear fraction.
b: urea-extract of sheared whole cells.

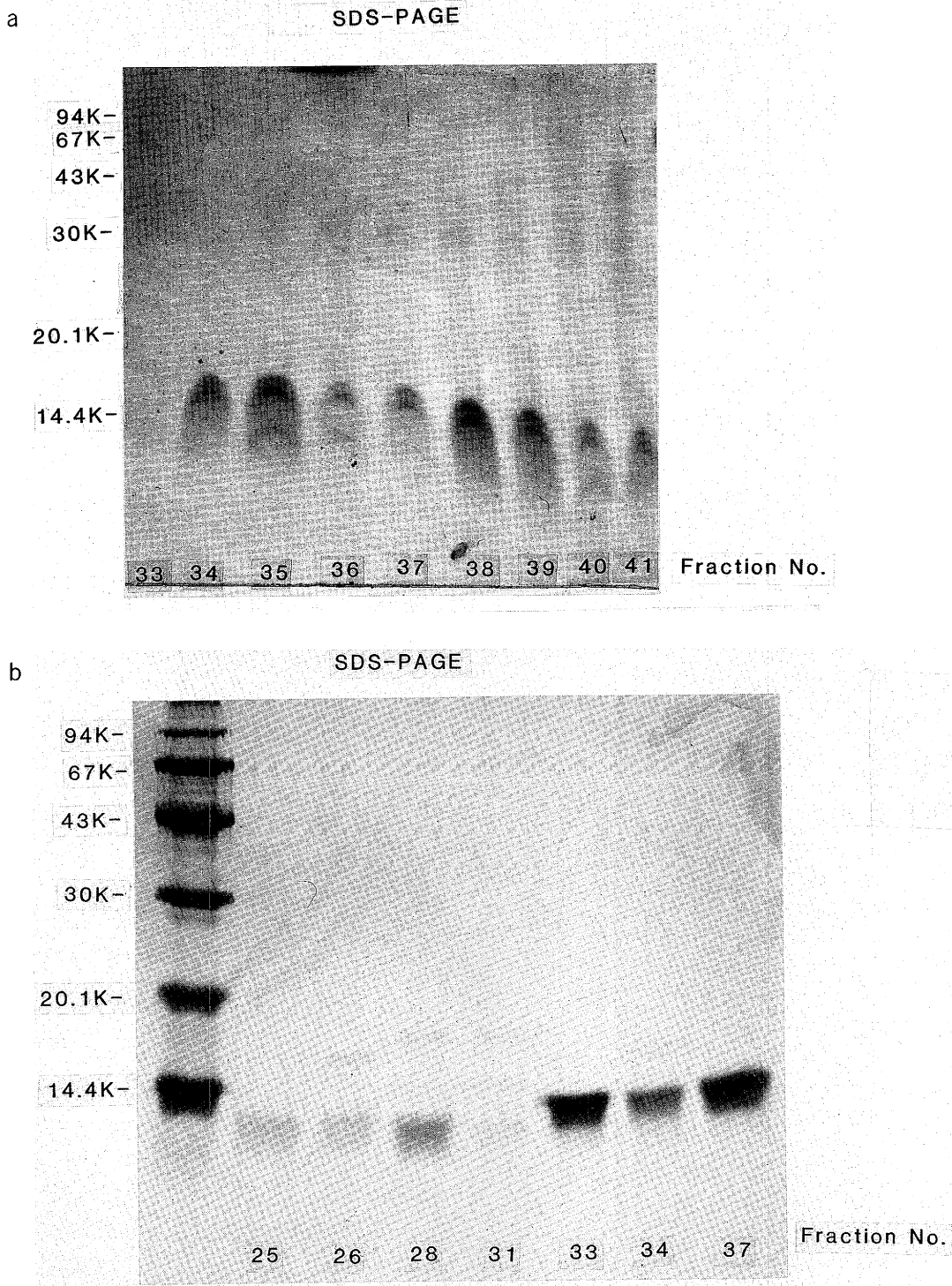


Fig. 4. SDS-PAGE Profiles of fractions eluted from reversed phase column chromatography.
a: shear fraction, b: urea-extract of sheared whole cells.

(a)

Fraction 35	Ala	Lys	Gly	Gln	Ser	Leu	Gln	Asp	Pro	Phe	Leu	Asn	Ala	Leu	---	---	Glu	---	Ile	Pro
	Val	Ser	Ile	Tyr	Leu	Val	Asn	Gly	Ile	Lys										

(b)

Fraction 33	Ala	Lys	Gly	Gln	Ser	Leu	Gln	Asp	Pro	Phe	Leu	Asn	Ala	Leu	---	---	Glu	---	Ile	Pro
Fraction 37	Ala	Lys	Gly	Gln	Ser	Leu	Gln	Asp	Pro	Phe	Leu	Asn	Ala	Leu	---	---	Glu	---	Ile	Pro
	Val	Ser	Ile	Tyr	Leu	Val	Asn	Gly	Ile	Lys										

Fig. 5. Comparison of N-terminal amino acid sequences of the fractions eluted from reversed phase column chromatography.
a: shear fraction, b: urea-extract of sheared whole cells. Note the identical N-terminal amino acid sequences among the preparations with different origins.

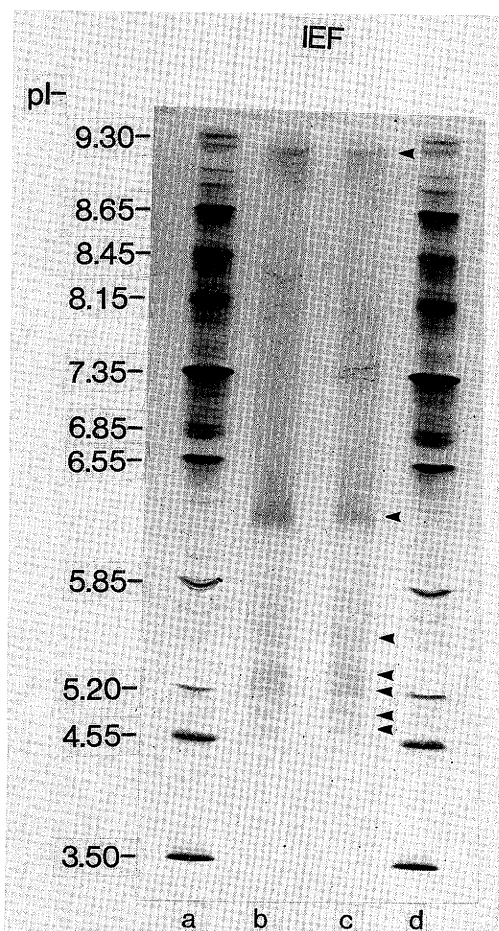


Fig. 6. Iso-electrofocusing profile of HPLC-purified 12 kDa protein.
lanes a, d: pI marker protein kit (Pharmacia),
b, c: fraction 35.

chromatographed using the same procedure as for the sample of shear fraction. The chromatogram of a reversed phase column is shown in Fig. 3b. Figure 4b shows a SDS-PAGE profile of the peak fractions. Samples taken from two major peak fractions (No. 33 and 37) were loaded on a protein sequencer and results are shown in Fig. 5b. Apparently the N-terminal amino acid sequences of the proteins taken from these two major peaks are identical. Surprisingly, we found proteins with the same N-terminal amino acid sequences among preparations with different origins, that is, among the peak elution fractions from the shear fraction, cell-wash and urea-extract of sheared cells (Fig. 5). When the samples taken from fraction No. 35 (Fig. 3a) and 33 (Fig. 3b) were loaded onto a Ampholine PAG PLATE (LKB) after treatment with 6M urea, numerous bands appeared at pIs, 6.3, 5.3 (Fig. 6) as previously reported for the soluble haemagglutinin of *V. cholerae* O1 (Finkelshtein and Hanne, 1982).

DISCUSSION

CS resin is used for the purification of filamentous haemagglutinin (FHA) of *Bordetella pertussis* in The Chemo-Sero Therapeutic Research Institute, Kumamoto, Japan. Here we have shown that this resin is useful for the purification of fimbrial antigens from *V. cholerae* O1. In addition, the fimbrial subunit of *V. cholerae* O1 was shown to form a multimer with various chemical modifications and various molecular weights.

The different SDS-PAGE profiles between shear fraction and urea-extract of sheared whole cells (Fig. 4) suggest the presence of two types of fimbrial antigen: an extracellular form (fimbriae) and an intracellular form (possibly a cytoskeletal protein), although the subunits of two types have the same N-terminal amino acid sequences.

HA activity of purified fimbriae from K23-7 strain by sucrose gradient centrifugation showed sensitive to both D-mannose and L-fucose (Ehara *et al.*, 1987), however fimbrial preparation purified by CS resin affinity chromatography after treatment with urea showed resistant to both mono-saccharides. This discrepancy may derive from a certain structural change of the fimbrial molecule after treatment with urea. There is also another possibility that minor contamination of cell-associated haemagglutinin in the fimbrial preparation may affect the HA activity.

Although we succeeded in the purification of fimbrial subunit of *V. cholerae* O1 by affinity chromatography, it is still uncertain how fimbriae interact with cell-associated haemagglutinin. In this experiment, we treated the fimbrial preparation with 8 M urea to dissociate the fimbriae from the other components. Now a new trial to isolate fimbriae-associated proteins using the same affinity column chromatography is in progress.

We have shown the cross reactivity in fimbrial subunits independent of biotype and serotype (Ehara *et al.*, 1987). We have also proved that the fimbrial subunit of *V. cholerae* O1 is recognized by the blood sera from convalescent cholera patients (in press). For the purpose of developing a new component vaccine against cholera, the fimbrial antigen of *V. cholerae* O1 seems to be one of suitable candidates.

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