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Interaction of a rat intestinal brush border membrane glycoprotein with type-1 fimbriae of *Salmonella typhimurium*

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Abstract

Type-1 fimbriated *Salmonella typhimurium* was found to adhere to rat intestinal brush border membrane in a mannose sensitive manner. The maximum binding of the purified fimbriae observed with the rat illeal enterocytes was inhibited by 69.2% in presence of D-mannose. Brush border membrane from rat illeum was isolated, delipidified, solubilised and fractionated by affinity chromatography on type-1 fimbriae coupled Sepharose CL 4B column. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the material eluted from the column with D-mannose revealed a single band of molecular weight 60 kDa. The direct binding of this affinity eluted glycoprotein to the purified type-1 fimbriae was demonstrated by a modified Western blot experiment. Our findings suggest that the 60 kDa glycoprotein may serve as a receptor for the type-1 fimbriae in the rat intestinal brush border membrane and thereby may help in mediating bacterial adherence to the host epithelium. (Mol Cell Biochem **158:** 125–131, 1996)

Key words: Salmonella typhimurium, fimbriae, mannose, glycoprotein, receptor

Introduction

Salmonellosis is a continuing problem in man and animals. Infectious and noninfectious infantile diarrhoea are leading causes of childhood morbidity and mortality especially in communities where malnutrition and deficient hygiene prevail [1, 2]. Studies in developed and under developed countries indicated that Salmonella was the etiologic agent of acute diarrhoea in children and *Salmonella typhimurium* was the most frequently isolated strain [3]. This microorganism infects humans of all ages [4] and generally causes a self limiting gastroenteritis through an invasive mechanism although bacteremia may occur in children [5].

S. typhimurium attachment is the prerequisite and first step in a biological continum which leads to penetration of enterocytes with subsequent invasion into deeper tissues as well as the blood stream [6–8]. It has been reported that type-1 fimbriated strains of *S. typhimurium* adhered in significantly higher numbers to isolated rat enterocytes than nonfimbriated strains and the adherence has been found to be inhibited by D-mannose and α-methyl-D-mannoside [9]. The molecular mechanisms involved in this process are not yet clear, however the direct evidence for the role of mannose sensitive adhesions in pathogenic process can only be provided by studying interaction with specific intestinal receptor(s). In this paper, we have reported the identification of the rat intestinal brush border membrane glycoprotein that interacts with the type-1 fimbriae of a virulent strain of *S. typhimurium*, the known classical pathogen for rat. This glycoprotein may play a key role in the in vivo attachment of the bacteria to the epithelial surface.

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Materials and methods

Chemicals

Sepharose CL 4B, acrylamide, bisacrylamide and all the molecular weight markers used for gel electrophoresis were the products of Pharmacia Chemicals, Uppsala, Sweden. Fluorescein conjugated swine immunoglobulins to rabbit immunoglobulins and Horse Radish Peroxidase conjugated swine immunoglobulins to rabbit immunoglobulins were purchased from Dakopatts, Denmark. All other chemicals used were of analytical grade.

Bacterial strain and growth conditions

Seed culture of *S. typhimurium* 11828 (procured from CRI, Kasauli, India) was prepared in BHI broth for 6 h. For the purification of fimbriae, the bacteria were grown on BHI agar for 18 h at 37°C in 1 l Roux bottles (conditions which are optimal for the growth of bacteria expressing mannose specific activity, as determined by agglutination of guinea pig erythrocytes both in presence or in absence of 1% D-mannose). All cultures were tested for type-1 fimbriae by agglutination with antifimbrial serum before their use in the assays.

Purification of type 1 fimbriae

Type-1 fimbriae were isolated by the mechanical agitation of bacterial suspension followed by differential centrifugation and ion exchange chromatography on Mono-Q HR-5/5 column (Pharmacia) in the FPLC system [10]. The purified fimbriae were tested for its agglutinating activity with guinea pig erythrocytes both in presence or in the absence of D-mannose.

Hemagglutination (HA) assay and inhibition of HA

HA assays were performed by the serial dilution technique in Takatsy microtitrator (Laxbro Mfg, Co, India) with 1% (v/v) guinea pig erythrocytes suspension in saline [11]. HA titres (units) were reported as the reciprocal of the highest dilution of bacteria or protein giving visible agglutination. The effect of Ca^{2+} and Mn^{2+} (0–15 mM) on HA activity of type-1 fimbriae were studied at 25°C and pH 7.4. The inhibition of HA activity in presence of different sugars was carried out by the method of Basu *et al.* [11].

Immunological methods

New Zealand white rabbits were immunized with FPLC purified type-1 fimbriae by the method of Ghosh *et al.* [10]. The antisera were tested by the enzyme linked immunosorbent assay (ELISA) [12]. To remove any possibility of interference of antibodies to nonfimbriated surface components, the antisera was absorbed five times with the defimbriated bacterial cells (the fimbriae were removed by heating the bacterial cells suspended in 75 mM NaCl at 65°C for 30 min) [13]. The absorbed serum was found to agglutinate the bacteria (11828), the purified fimbriae but not the defimbriated bacterial cells.

Preparation and fractionation of brush border membrane from rat intestine

Brush border membrane (BBM) of rat intestine was isolated from the Albino rats (Wistar strain) of either sex by the method of Kessler *et al.* [14]. Delipidification of BBM was done with the addition of 20 volumes of ethanol/acetone (1:1) followed by centrifugation at 2000 rpm for 10 min. The membrane pellet was suspended in 10 mM Tris buffer (pH 8.0) containing 150 mM NaCl, 1.5 mM NaN_3, 1 mM PMSF and 0.5% Triton X-100, incubated for lh at 4°C and centrifuged at 30000 rpm for 1 h at 4°C. The supernatant (BBMS) was dialysed extensively against 10 mM Tris buffer (pH 7.0) containing 1.5 mM NaN_3 and 1 mM PMSF.

Bacterial adherence to immobilized BBM

A 96 well flat bottomed enzyme immunoassay plate was coated overnight with 0.1 ml BBM (50 µg protein/ml) in 50 mM carbonate buffer (pH 9.6). The coated wells were washed thrice with 20 mM Tris buffer (pH 7.2) containing 150 mM NaCl (TBS). After blocking of residual binding sites by incubation of the coated wells with 1% BSA in TBS (BSA-TBS), a sample of type-1 fimbriated *S. typhimurium* (100 µl containing 10^8 cells) was added to the wells, incubated for 1 h at 37°C and washed. The control wells received only bacteria. Additionally for inhibition studies, the bacterial cells mixed with 2% solution of D-mannose, was added to the BBM coated wells. This was followed by further incubation for 2 h with rabbit antifimbrial serum in a dilution of 1:2000 in BSA-TBS at 37°C followed by standard ELISA procedure [12].

Enterocyte isolation

Enterocytes were isolated from three different parts of intestine i.e. duodenum, jejunum and illeum by the method of

Pinkus [15]. Viability of the cells was determined by the trypan blue exclusion test. More than 90% of the enterocytes were found to be viable.

Assay for binding of type-1 fimbriae to enterocytes

Freshly isolated enterocytes from duodenum, jejunum and illeum were used separately in the binding assay. Enterocytes (3×10^5 cells/ml) in TBS were incubated with purified fimbriae (1.5 mg protein/ml) or fimbriae preincubated with different sugars (2.0% each) for 1 h at 37°C. The unbound fimbriae were washed off (thrice) with TBS. Enterocytes with bound fimbriae were further incubated with rabbit antifimbrial serum (primary antibody) in a dilution of 1:250, incubated at 37°C for 1 h and later washed to remove unbound antibody. This was followed by incubation with Fluorescein conjugated swine anti rabbit immunoglobulin (secondary antibody) in a dilution of 1:50. After extensive washing, paraformaldehyde solution (0.5%) was added to the cells. The percent binding of enterocytes to the type-1 fimbriae in absence or in the presence of different sugars was analysed by flow cytometry. At least 10000 cells were acquired and analysed on LYSIS II software of FACSCAN (Becton Dickenson, USA). For analysis boundaries were set on control histograms to include at least 98% events and then the percent increase in log fluorescence intensity was calculated for other samples. All control experiments run in parallel included (i) enterocytes treated with both primary and secondary antibody (ii) cells treated with secondary antibody.

Analytical method

SDS-PAGE was carried out in vertical slab gel by the system of Laemmli [16] with suitable protein markers.

Affinity chromatography

Purified fimbriae were coupled to cyanogen bromide activated Sepharose CL 4B (1 mg protein/ml of Sepharose) by the method of March *et al.* [17]. Under these conditions, 85% of the fimbriae were bound to the Sepharose based on the protein estimation of fimbrial suspension before and after coupling, by the method of Lowry *et al.* [18]. The dialysed 22 ml BBMS (0.9 mg/ml) prepared from the illeum was loaded on a fimbriae – Sepharose column (1.5 × 9 cm) equilibrated with 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Triton X-100 and 0.02% NaN_3 (buffer A) at 4°C. The column was washed until the optical density of the effluent became less than 0.01 against buffer A. The bound material was eluted with 500 mM D-mannose in buffer A (buffer B) at room temperature. The eluted material was pooled, dialysed extensively against buffer A devoid of Ca^{2+} and Triton X-100 and concentrated by ultrafiltration.

Direct binding of type-1 fimbriae to affinity purified glycoprotein

After electrophoresis of the affinity purified glycoprotein (50 µg) on SDS polyacrylamide gel (7.5%) under reducing conditions [16], the protein band was electrophoretically transferred onto nitrocellulose paper by the method of Towbin *et al.* [19] After transfer the nitrocellulose paper strips were placed in BSA-TBS overnight at 4°C, washed three times with TBS and incubated with fimbrial antigen (100 µg protein/ml) for 2 h at 37°C both in presence or in absence of 2% D-mannose. After extensive washing with TBS containing 0.05% Tween-20 (TBST), the nitrocellulose paper was treated with rabbit antifimbrial serum in a dilution of 1:250 in BSA-TBS, washed thoroughly with TBST followed by addition of HRP-conjugated swine anti rabbit immunoglobulin in a dilution of 1:1000. After 1 h incubation at 37°C, the nitrocellulose paper was washed extensively and placed in 0.05% 3,3′-Diaminobenzidine tetrahydrochloride in TBS containing 10 µl H_2O_2 for 30 min. Reaction was stopped by washing the nitrocellulose strips with water.

Determination of protein

Proteins were estimated by the method of Lowry *et al.* [18] and Bradford [20].

Results

S. typhimurium (10^9 cells/ml) was found to agglutinate guinea pig erythrocytes. The HA activity of the bacteria was found to be inhibited by 1% D-mannose which suggests the presence of type-1 fimbriae on the bacteria. The purified fimbriae were eluted out in the last peak at 750 mM NaCl in 10 mM Tris buffer at 16 min from Mono Q anion exchange column (Fig. 1) in FPLC system [10]. The fimbriae agglutinated guinea pig erythrocytes and the titer was found to be 1:5120. The effect of Ca^{2+} and Mn^{2+} (0–10 mM) on the HA activity of the purified fimbriae were studied. It has been found that in the presence of 5 mM Ca^{2+}, HA titer increased by two fold. No significant effect on HA activity of fimbriae was observed in presence of Mn^{2+}. Higher concentration of both Ca^{2+} and Mn^{2+} was found to be inhibitory. The HA activity of the fimbriae was found to be inhibited in presence of D-mannose which confirmed the type-1 nature of the purified fimbriae. Hyperimmune rabbit antitype-1 fimbrial serum was found to

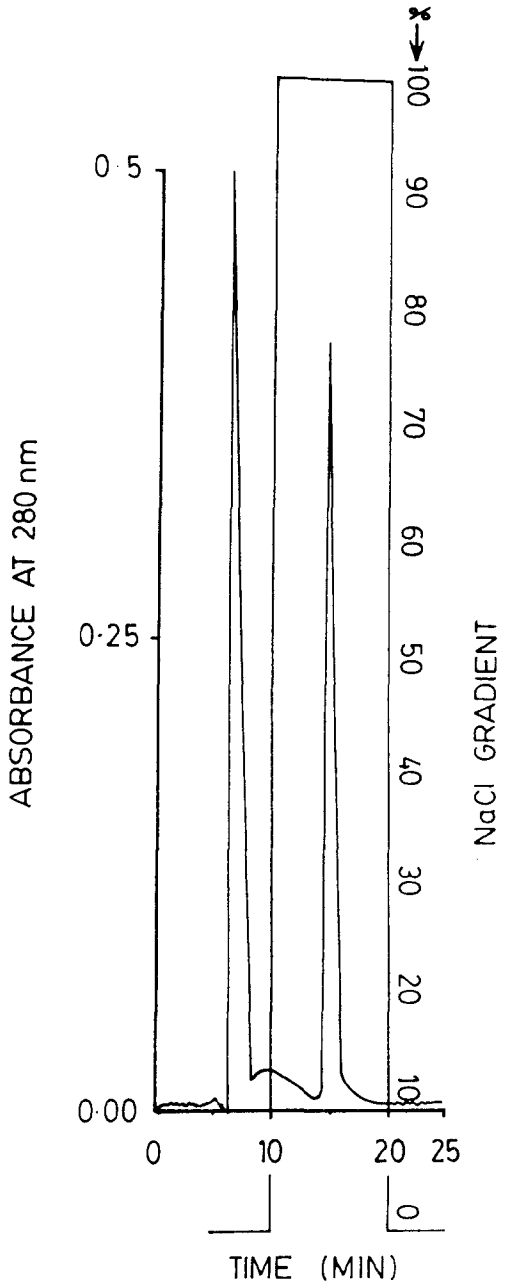

Fig. 1. Elution profile of the Mono-Q column for type-1 fimbriae. Semipure fimbriae obtained after ultracentrifugation were loaded onto Mono-Q column. Elution was achieved with 10 mM Tris -HCl buffer (pH 7.0) for 10 min followed by the same buffer containing 750 mM NaCl for 10 min at 0.75 ml/min. The purified fimbriae were eluted out in the last peak at 750 mM (100%) NaCl concentration in 16 min [10].

have a titer of 1:10000 with 10 μg/ml of fimbriae, whereas the titer of the preimmune serum under the same condition was < 1:8.

Total protein content found from BBM of intestines (26 gm) of 12 rats (each weighs 30 gm) was 50–60 mg. Membrane purity was established by assaying one of the marker enzyme (alkaline phosphatase) activity. The adherence

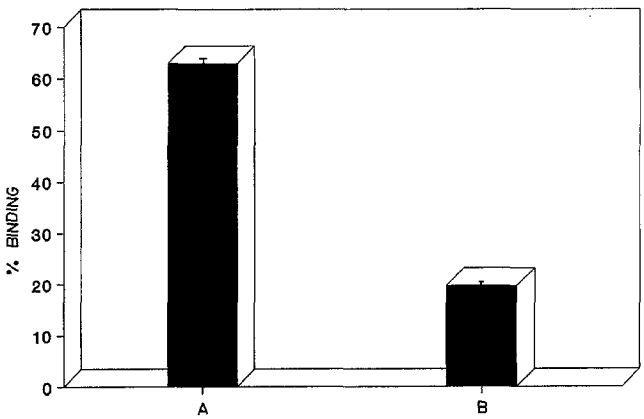

Fig. 2. Enzyme-linked immunosorbent assays of attachment of type-1 fimbriated *S. typhimurium* to immobilised BBM using rabbit antifimbrial serum (A) in absence of mannose (B) in presence of mannose.

(62.9% ± 1.0%) of *S. typhimurium* to brush border membrane was found to be reduced to 19.7% ± 0.4% in presence of 2% D-mannose (Fig. 2). Flowcytometric analysis indicated 94.7 and 78.6% binding of the purified fimbriae with the enterocytes from illeum and jejunum respectively (Fig. 3). No binding was observed with the enterocytes obtained from duodenum. Subsequently the inhibition of binding of purified fimbriae with illeal enterocytes in presence of different sugars was evaluated and the results showed 69.2, 7.3 and 1.3% inhibition in presence of D-mannose, D-glucose and D-galactose (2.0% of each) respectively (Fig. 4). SDS-polyacrylamide gel electrophoretic pattern of BBM and BBMS is shown in Fig. 5. The affinity chromatography (Fig. 6) eluted material with buffer B revealed a single band of Mr 60 kDa in 10% SDS-PAGE (Fig.7). The yield of the glycoprotein was found to be 169 μg from 20 mg of solubilised BBM. The interaction of purified type-1 fimbriae to the fimbriae-Sepharose affinity chromatography purified glycoprotein was visualised as darkly staining band on the nitrocellulose paper (Fig. 8), whereas in presence of 2% mannose no band was observed.

Discussion

In this paper we have demonstrated the specific binding of type-1 fimbriated *Salmonella typhimurium* to rat intestinal brush border membrane in a solid phase assay. The binding was found to be inhibited by D-mannose, which confirms that mannose is a part of the BBM component(s), required for binding of *S. typhimurium*, expressing type-1 fimbriae. This finding is in good agreement with our data on the percent binding of type-1 fimbriae to enterocytes in absence and in presence of D-mannose. Our results indicate that mannose sensitive fimbriae played a role in the attachment of *S. typhimurium* to rat intestinal BBM which had also been re-

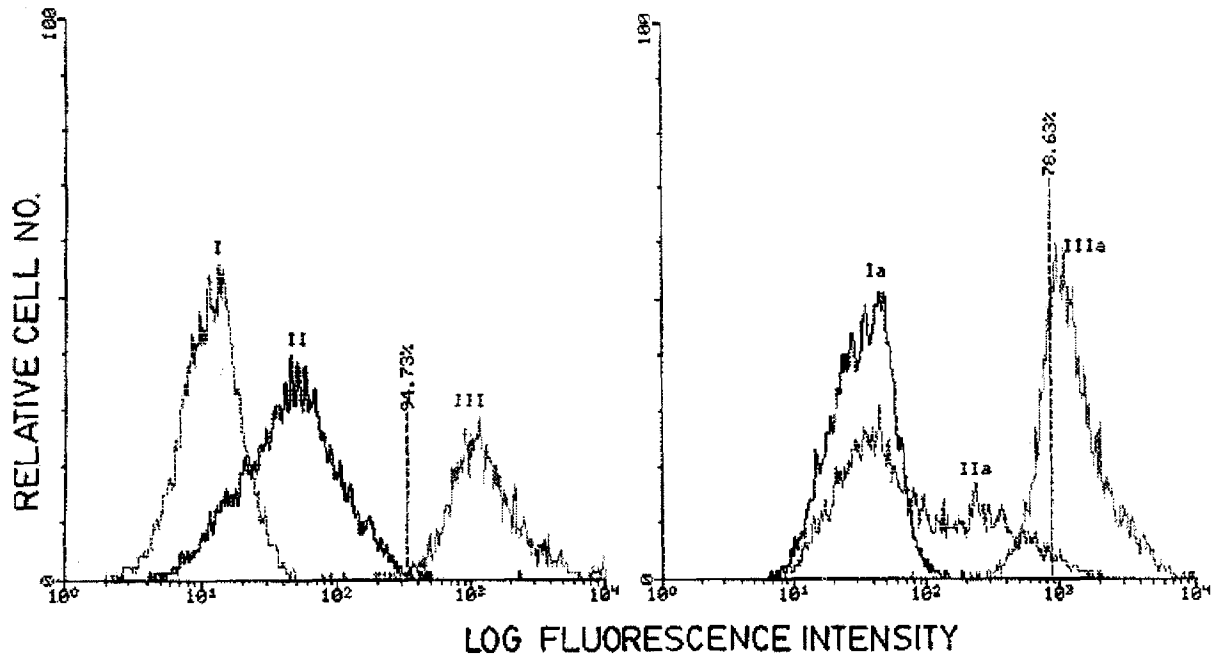

Fig. 3. FACS analysis of binding of enterocytes to type-1 fimbriae. (A) Illeal enterocytes treated with (I) secondary antibody. (II) primary and secondary antibody (III) purified fimbriae, primary and secondary antibody. (B) Jejunum enterocytes treated with (Ia) secondary antibody (IIa) primary and secondary antibody (IIIa) purified fimbriae, primary and secondary antibody.

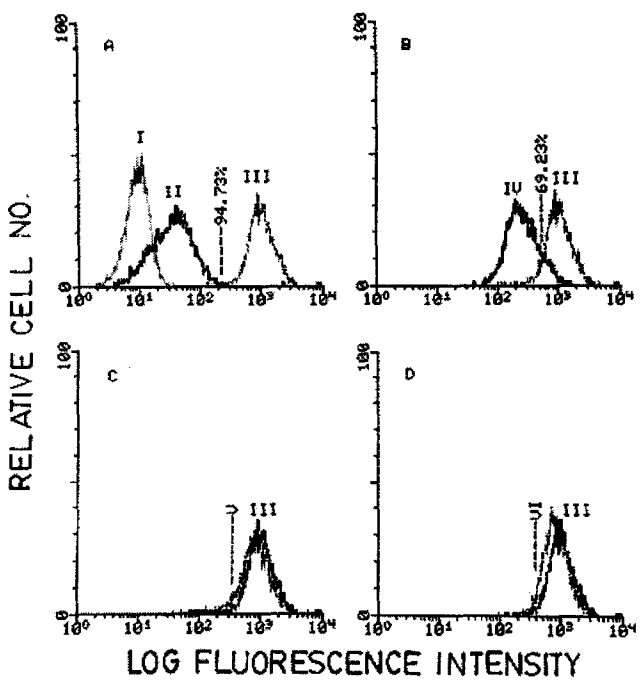

Fig. 4. FACS analysis of binding of illeal enterocytes to type-1 fimbriae in absence or in presence of different sugars. (A) Enterocytes treated with (I) secondary antibody (II) primary and secondary antibody (III) purified fimbriae primary and secondary antibody. (B) Enterocytes treated with (IV) purified fimbriae pretreated with mannose (V) purified fimbriae pretreated with galactose (VI) purified fimbriae pretreated with glucose, followed by primary antibody and secondary antibody respectively.

ported earlier [8, 9]. It has been postulated that the combining site of type-1 fimbrial lectin of *E. coli* corresponds to the size of a trisaccharide. The presence of a hydrophobic binding region adjacent to the binding site of *E. coli* – type-1 fimbriae has also been indicated, however the major binding site of *S. typhimurium* is considerably different and devoid of hydrophobic region [21]. In general, it appears that mannose specific bacteria preferentially bind structures found in short oligomannose chains of N-linked glycoproteins, which are common constituents of many eukaryotic cell surfaces [22], The N-linked oligo-mannosides specific for type-1 fimbrial lectin or adhesion probably reside on more than one type of molecule on the eukaryotic cell surfaces for example, type-1 fimbriae of *E. coli* bound to three glycoproteins derived from the surface membrane of human polymorphonuclear leukocytes [23].

Several studies have been carried out regarding the receptor of type-1 fimbriae of enterobacteriaceae [22–25]. It has also been shown that the receptors for *E. coli* K-12 (K 88 ab) which are responsible for adhesion to mouse mucus and brush border membrane, appear to be glycoproteins which very likely contain D-galactosamine like residues [24]. Studies on other adhesion receptor systems such as the mannose sensitive type-1 *E. coli* adhesion; the α-D-Gal-[1–4]-β-D-Gal binding adhesins and the glycolipid binding adhesins [26–29] have revealed that the galactosamine like component is almost present as a part of complex carbohydrate moiety.

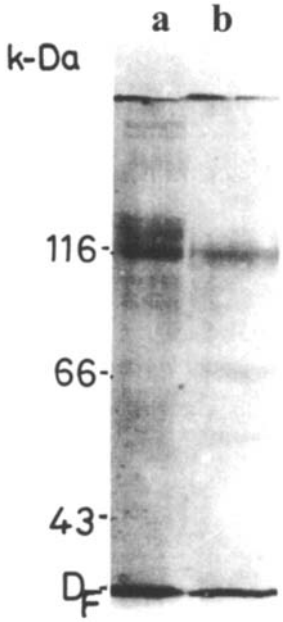

Fig. 5. Electrophoretic pattern of BBM and BBMS from rat intestine on SDS-PAGE (7.5%). The procedure described by Laemmli [16] was followed. Lanes (a) BBM (b) BBMS. Molecular weights of the protein standards are also indicated.

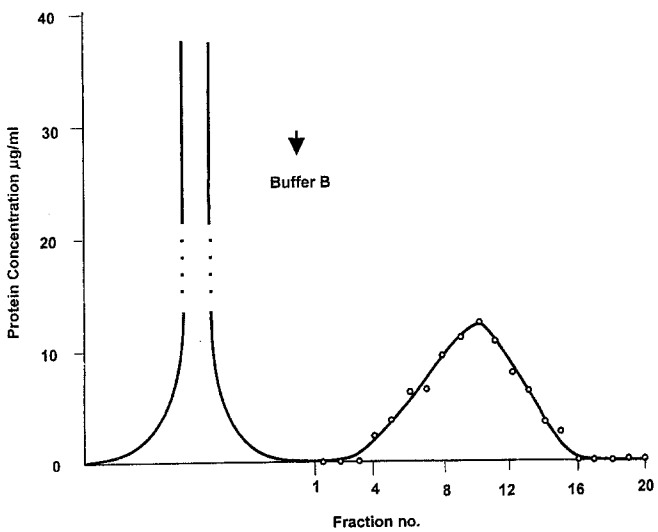

Fig. 6. Elution profile of the affinity column. 22ml dialysed Triton X-100 soluble BBM (0.9 mg/ml) was applied to fimbriae-Sepharose CL4B column (1.5 × 9 cm). After washing the column with buffer A, until the absorbance of the effluent at 280 nm was less than 0.01, 20 test tubes each containing 1 ml fraction of the eluate were eluted with buffer B at room temperature. Protein concentration was monitored by Bradford assay [20].

The aim-of this study was to isolate the rat intestinal brush border membrane component(s) that is involved in the binding of type-1 fimbriae of *S. typhimurium*. For this purpose we used affinity chromatography on immobilised type-1 fimbriae. The 60 kDa protein eluted with D-mannose suggests

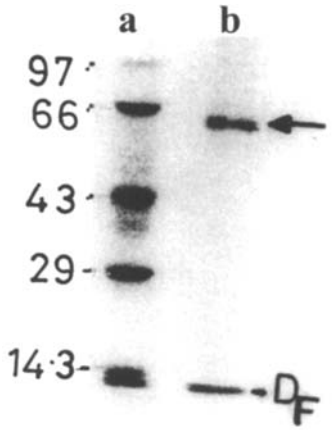

Fig. 7. Electrophoretic pattern of affinity eluted glycoprotein on SDS-PAGE (10%). Lanes (a) Molecular weight markers, phosphorylase b (97 kDa), BSA (66 kDa) ovalbumin (43 kDa), Carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa) (b) affinity purified glycoprotein (15 ug).

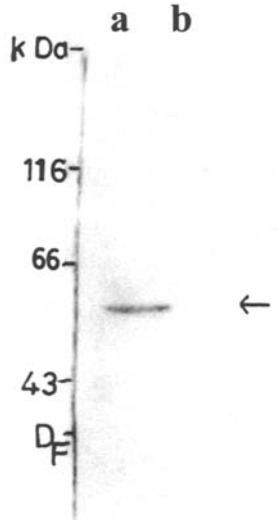

Fig. 8. Direct interaction of affinity purified glycoprotein with type-1 fimbriae. Nitrocellulose strips treated with (A) purified fimbriae in absence of D-mannose (B) purified fimbriae in presence of D-mannose followed by rabbit antifimbrial serum and secondary antibody.

the glycoprotein nature of it as well as the importance of its mannose residues in binding the fimbriae. Hence it can be concluded that a 60 kDa glycoprotein exists on rat intestinal BBM for binding type-1 fimbriae of *S. typhimurium*, which may help in mediating bacterial adherence to the host epithelium and may act as a receptor for type-1 fimbriae of the bacteria. Further characterization of the BBM glycoprotein is required. The detailed study of this glycoprotein will possibly lead to a better understanding of the basic molecular mechanism involved in the bacterial interaction with the host cell. The information provided with this study will certainly

strengthen our knowledge regarding the pathogenesis of the disease caused by this microorganism.

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