Structural and Functional Changes in Rabbit Ileum by Purified Extracellular Phospholipase A of *Salmonella newport*

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**ABSTRACT.** As phospholipases of *Salmonella* species may play a role in the pathogenesis of gastrointestinal tract diseases. *Salmonella newport*, the causative agent of infantile diarrhoea was examined for the production of phospholipase. The enzyme was purified by gel filtration chromatography and was found to be a protein of molar mass ranging from 43 to 67 kDa. The purified enzyme alone or in combination with organisms, produced both structural and functional changes in rabbit ileum, contributing towards pathogenesis of diarrhoea due to this organism.

The genus *Salmonella* consists of Gram-negative microorganisms that inhabit the intestine of a large number of vertebrates. These infect man, causing enteric fever and gastroenteritis. It is now believed that the virulence of *Salmonella newport*, the causative agent of infantile diarrhoea, is multifactorial. Some salmonellae are well known for their lipolytic activity, hydrolyzing lipids and phospholipids. This activity is attributed to phospholipases (Bhandari and Asnani 1989; Bal 1983). Keeping in view the clinical importance of *S. newport* in gastrointestinal infections and considering phospholipase production as one of the virulence factors, attempts were made to test purified phospholipase A of *S. newport* for any enterotoxic activity and what structural and functional changes it produces in rabbit ileum.

**MATERIAL AND METHODS**

**Source of phospholipase.** For the production of extracellular phospholipase, a strain of *S. newport* (6,8:ch1,2) obtained from the Central Research Institute, Kasauli, was used. The phospholipase was purified from the supernatant of an overnight broth culture of the organisms (Neena 1987), using salt precipitation followed by gel filtration chromatography (Campbell *et al.* 1971). The term enzyme shall be used for phospholipase from here on. Purified enzyme was analyzed biochemically for protein, sugars (Miller 1959), RNA (Herbert and Phipps 1971) and DNA (Burton 1956). Molar mass was determined by SDS-PAGE, using Laemmli's discontinuous buffer system (Laemmli and Farve 1973).

**Coagglutination test.** To determine qualitatively the enterotoxigenicity of *S. newport*, coagglutination test was used (Ronnberg and Wadstrom 1983). Briefly, equal volumes (25 μL) of cell lysates, obtained by mixing a loopful of 1-d-old growth in saline, containing polymyxin B and coagglutination reagent (Anti-LT coated *Staphylococcus aureus* strain Cowan 1)were mixed on phadebact cellulose paper cards and observed for coagglutination at room temperature. Appearance of clumps within 2 min was considered as positive for enterotoxin production. Known enterotoxigenic as well as nonenterotoxigenic strains of *E. coli* were also included in the test as a positive and negative control, respectively.

**Biological assay for enterotoxic activity of the enzyme.** Rabbit ileal loop method of Kasai and Burrows (1966) was used. Rabbits averaging 1.5–2.0 kg body mass were used and fasted up to 24 h but were given water ad libitum. Before the operation, the rabbit was anesthetized, incised and the small intestine was exposed, ligated into loops, each measuring 60–70 mm followed by 10–20 mm small segments alternatively acting as locks. Each test and control loops received 1 mL of test sample and sterilized broth, respectively. The ligated loops were repositioned and the abdomen was sutured. The
rabbit was observed for 1 d and then sacrificed. The length of each loop was recorded and the volume of fluid accumulated in it was measured. The loops were opened and examined for gross lesions and histopathological studies. Gut dilatory response (GDR) was calculated as follows:

\[ \text{GDR} = \frac{V}{L}, \]

where \( V \) is the volume of fluid accumulate (mL) and \( L \) length of loops (mm).

GDR > 1 was considered positive for enterotoxic activity.

(i) Supernatant broth culture, (ii) heated supernatant, (iii) enzyme, (iv) heated enzyme, (v) organisms, (vi) enzyme + supernatant, (vii) enzyme + organisms, (viii) enzyme + antienzyme serum (one unit) were tested for toxicity.

Raising antiserum against the enzyme. Rabbit serum was checked for antibodies against the enzyme before raising antiserum. Rabbits were immunized with three doses of purified enzyme (1–2 mg protein) given subcutaneously at weekly intervals and the last injection was given intravenously. The serum was collected and checked for antibodies against the enzyme by CIEP.

Neutralization of enzyme by antienzyme serum. Two fold dilutions ranging from 2 to 256 of antiserum were prepared in normal saline and 200 \( \mu \)L of each dilution, was combined with 200 \( \mu \)L of enzyme (1–2 mg protein per mL). The tubes were incubated at 37 °C for 30 min and subsequently put into wells made in egg-yolk plates (Haberman and Harde1972). The enzyme and neat serum were used as controls. The highest dilution of antiserum, completely neutralizing the enzyme activity (no zone of clearance produced around wells in egg-yolk plates) was recorded and designated as one unit of antienzyme.

Biochemical analysis of fluid. The fluid accumulated in the loops was assayed for sodium, potassium (Hawk 1954), chloride (Schales 1941) and hydrogen carbonate (Van Slyke 1953).

Histopathological examination. Tissue samples of tests and control loops were preserved in 10 % formaldehyde for histopathological studies. The tissue sections were stained with hematoxylin–eosin and examined under the microscope.

RESULTS

The purified enzyme was a protein with molar mass ranging from 43 to 67 kDa. The lyzate gave a positive coagglutination due to aggregation of coated staphylococci. Rabbit ileal loop assay was performed to establish whether purified enzyme alone or in combination as described above, would induce gut dilatory response. It was observed that (Table I) the GDR was quite evident in case of supernatant and \( S. \) newport culture. The enzyme gave a GDR of 0.79 (mean value). Both heated supernatant and heated enzyme failed to give any response. The combination of "enzyme and supernatant" and "enzyme and organisms" gave a GDR of 1.28 and 1.23, respectively. The enzyme was neutralized with antienzyme antibodies, did not give such response and was as good as the control. The enzyme activity was neutralized by its homologous antiserum at a dilution of 1:16.

Biochemical analysis of accumulated fluid in different test loops revealed increased concentrations of electrolytes, viz. sodium, potassium, chloride and hydrogen carbonate as compared to the control. Greatest increase was noticed with hydrogen carbonate followed by chloride, sodium and potassium (Table II).

Histopathological examination of the test loops inoculated with purified enzyme showed desquamation of epithelial lining and mononuclear cell aggregation in the lamina propria, some of which were degenerated, leaving behind karyorrhectic nuclear debris. Submucosal edema was evident in one section, which also showed subserosal edema along with mild scattered mononuclear cell infiltration. Small focal aggregates of mononuclear cells were also seen just below the serosal lining (Fig. 1A, B). The loops inoculated with organisms showed, in addition to the above, changes at places, the clumps of desquamated epithelial cells having bacterial colonies. The muscular layers had vacuolations along with mild mononuclear cell infiltration in the tunica muscularis (Fig. 1C). Similar changes were observed in the test loops inoculated with supernatant, enzyme + supernatant and enzyme + organisms (Figs 1D, 2A,B). The loops inoculated with heated supernatant, heated enzyme or enzyme treated