

## Proteolytic and lipolytic properties of endotoxins (enterotoxins) produced by *Salmonella typhi* NCIM 5255, *Salmonella typhimurium* NCIM 2501 and *Shigella flexneri* NCIM 5265

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### Abstract

Endotoxin is a major component of the outer membrane (OM) of Gram-negative bacteria. It was observed that endotoxin plays an important role in the pathogenicity of Gram-negative bacterial infections. In humans, it is a potent mediator of a broad range of patho-physiological effects mostly in the gastrointestinal tracts. Hence, these are also referred as enterotoxins. These toxic activities like lethal toxicity, pyrogenicity, and tissue-necrotizing activity as well as many beneficial ones related to immunostimulation. This investigation involve the extraction of endotoxins from *Salmonella typhimurium* NCIM 2501, *Salmonella typhi* NCIM 5255, *Shigella flexnerii* NCIM 5265. Highest amount 23mg/mL of endotoxin was obtained from *Salmonella typhimurium*. Further the extracted endotoxin was used to check antibacterial activity on other bacteria, which showed good antibacterial activity against *Bacillus subtilis*. It also showed caseinase and lecithinase activity. Endotoxin extracted from *Salmonella typhimurium* had caseinase and lecithinase activity more as compared to that of *Salmonella typhi*, *Shigella flexnerii*.

### Keywords

Endotoxin  
*Salmonella*  
*Shigella*  
Enterotoxin  
Antibacterials  
Protease  
Lipase.

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### Introduction

Endotoxins are high-molecular weight complexes of lipopolysaccharides (LPS) which is the major component of bacterial cell wall (McCuskey and Urbaschek, 1996). Endotoxin is a heat stable toxic substance released by gram negative bacteria's after disruption of cell envelopes (Beutler and Rietschel, 2003; Bishop, 2005). The role of endotoxins in bacterial pathogenesis (Beutler and Rietschel 2003) and their chemical characterization as lipopolysaccharide (LPS) have been studied earlier (Raetz and Whitfield, 2002; Raetz *et al.*, 2007). Chemically, LPS consist of a hydrophilic polysaccharide covalently linked to a hydrophobic lipid portion which is termed as lipid A, which anchors the molecules in the outer membrane (OM) (Brandenburg and Wiese 2004). Endotoxins play a major role in human disease states that created interest to investigate the pathogenicity of the producing bacteria (Rietschel *et al.* 1996). Lipopolysaccharide found to be an important activator for the activation of immune system that leads to nonspecific inflammatory immune response (Buyse *et al.* 2007). The specific interaction of LPS with cells leads to activation of cells such as cellular proliferation of murine B-lymphocytes, the formation and secretion of bioactive chemical

mediators (cytokines) produced by murine or human monocytes, vascular cells and macrophages (Raetz 1993). LPS is mainly responsible for the pathogenesis and manifestation of particularly Gram-negative infection, that causes septic shock and are therefore it is termed endotoxins (Raetz 1993). The studies were carried out on the enzymes and genes responsible for the biosynthesis and export of lipopolysaccharide; for these studies the genetic information about the Gram-negative bacteria is required (Buyse *et al.* 2007). This investigation aims to study the effect of bacterial endotoxin on microorganism (especially those that constitute the normal flora of the gut).

### Material and Methods

#### Micro-organisms used

*Salmonella typhimurium* NCIM 2501, *Salmonella typhi* NCIM 5255, *Shigella flexnerii* NCIM 5265. The organism culture was directly obtained from NCIM Pune, India. The cultures were maintained on sterile peptone agar slant (Peptone 1%, NaCl 0.5%, Agar 2.5%).

#### Growth pattern

These organisms (*Salmonella typhimurium* NCIM 2501, *Salmonella typhi* NCIM 5255, *Shigella*

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flexnerii NCIM 5265) were grown in nutrient medium (Peptone 1%, Yeast extract 1%, NaCl 0.5%) at 37°C on shaker (120 r.p.m.) and absorbance was recorded at 530 nm at the interval of 30 min.

#### Toxin production

The 3 organisms were grown as in growth pattern studies but the incubation was for 5 to 6 hours only. The growth after the incubation period was centrifuged at 10000 x g for 10 minutes, supernatant was discarded and the biomass was washed with saline (0.85% NaCl) several times by centrifuging at 10000 x g for 10 minutes, each time to remove all medium components and cell debris. The cell pellet was suspended in 20 ml of the same saline solution and chilled at 4 °C. The suspension was homogenized by sonication, with vibra model VC.130PB sonicator, at 50 amplitude for 5 min. This was again centrifuged at 10000 x g for 10 minutes to remove all cell debris and the clear supernatant was used for further studies. The protein content of the suspension was determined by Lowry's method (Lowry *et al.* 1951).

#### Antibacterial activity

The antimicrobial activity was carried out by using *Bacillus subtilis* and *Micrococcus aureus* as a test organisms. The 3 wells of 3 mm diameter were prepared on each plate. In each well, endotoxin samples were added in 0.1ml, 0.2ml, 0.3ml, 0.4ml, 0.5ml.... so on. They were incubated for 24 hours at 37°C. After 24 hours incubation, zone of inhibition on nutrient agar plates were measured.

#### Determination of caseinase (proteolytic) and lecithinase (phospholipolytic) activity

In each plate of milk agar and egg yolk agar, 3 wells were prepared and in each well of milk agar plate and egg emulsion agar endotoxin samples were added in different concentrations. The plates were incubated for 24 hours at 37°C. After incubation zone of hydrolysis were measured and their mean was calculated.

Lecithinase (phospholipolytic) activity zone were measured by performing the soap test. In this test saturated solution of CuSO<sub>4</sub> was added on the each egg yolk agar (Peptone 1%, NaCl 0.5%, Agar 3%) plates for 20 minutes. The blue zones obtained were measured and their mean was calculated.

## Results and Discussion

*Growth pattern of Shigella flexnerii, Salmonella typhi and Salmonella typhimurium*

Growth cycle of *Shigella flexnerii* was completed

Table 1. Lecithinase and caseinase activity of *Salmonella typhi*

Amount of endotoxin (mg)	Lecithinase activity (zone of hydrolysis in mm)	Caseinase activity (zone of hydrolysis in mm)
1.5	2	1.5
2.5	3	3
4	5	6
5	8	10
6	10	15

Table 2. Lecithinase and caseinase activity of *Salmonella typhimurium*

Amount of endotoxin (mg)	Lecithinase activity (Zone of hydrolysis in mm)	Caseinase activity (zone of hydrolysis in mm)
5	3	3
9	5	6
14	9	10
18.5	12	13
23	18	19

Table 3. Lecithinase and caseinase activity of *Shigella flexneri*

Amount of endotoxin (mg)	Lecithinase activity (zone of hydrolysis in mm)	Caseinase activity (zone of hydrolysis in mm)
3.5	2	3
6.4	6	7
10	10	9
13	12	10
16	15	13

within 9 hours. After 6 hours late exponential phase was observed and at this phase endotoxins was extracted by sonication. Growth cycle of *Salmonella typhi* was completed within 10 hours. After 7 hours late exponential phase was observed and at this phase endotoxins was extracted by sonication. Growth cycle of *Salmonella typhimurium* was completed within 10 hours. After 9 hours late exponential phase was observed and at this phase endotoxins was extracted by sonication. It was observed that *Salmonella typhi* produced 6 mg/mL, *Salmonella typhimurium* produced 23 mg/mL and *Shigella flexneri* produced 16mg/ml of endotoxin.

*Lecithinase and caseinase activity of Salmonella typhi, Salmonella typhimurium and Shigella flexneri*

Endotoxin of *Salmonella typhi* shows strong caseinase activity than lecithinase at 1000±1 concentration. Endotoxin of *Salmonella typhimurium*

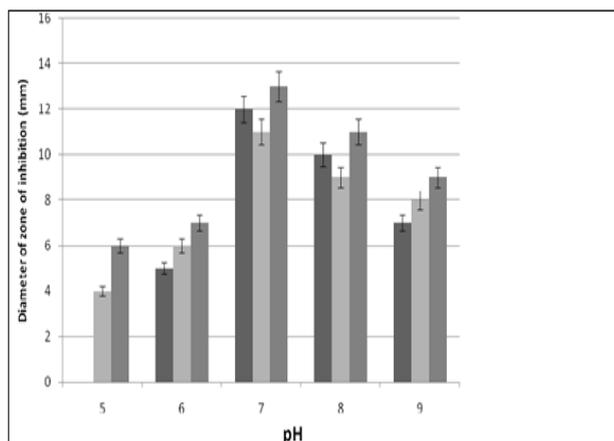


Figure 1. Effect of pH on antibacterial activity of endotoxins of (■) *Salmonella typhi*; (■) *Salmonella typhimurium* and (■) *Shigella flexnerii*, on *Bacillus subtilis*.

shows strong caseinase activity than lecithinase at  $1000 \pm 1$  concentration. Endotoxin of *Shigella flexneri* shows strong caseinase activity than lecithinase at  $1000 \pm 1$  concentration (Table 1, 2 and 3).

#### Effect of pH on endotoxin activity

Endotoxin of all 3 organisms shows minimum activity at pH below and above pH7, whereas it shows optimum activity at pH7 (Figure 1). This is a well known phenomenon where at a particular pH the macromolecule (especially proteins) shows minimum aggregation and thereby having maximum / optimum activity.

#### Effect of temperature on endotoxins activity

Endotoxin of all 3 organisms shows less activity at temperature below and above  $37^\circ\text{C}$ , optimum activity was observed near temperature  $37^\circ\text{C}$  (Figure 2). Temperature is known to affect the conformation of the endotoxin molecule and it is temporary changed at temperatures below and above the optimum temperature. This leads to loss of attachment of the molecule at the target site. Sometime if the temperature variation is too large, there could be a permanent loss of attachment of the endotoxin molecule.

#### Antibacterial activity

Endotoxin produced by *Salmonella typhi*, *Salmonella typhimurium*, *Shigella flexneri* shows good antibacterial activity at 6 mg, 23 mg and 16mg against *Bacillus subtilis* (Figure 1 and 2). This indicates the most potent endotoxin to be that of *Salmonella typhi*. The antibacterial activity against *Micrococcus aureus* wasn't very significant and hence the results were not recorded.

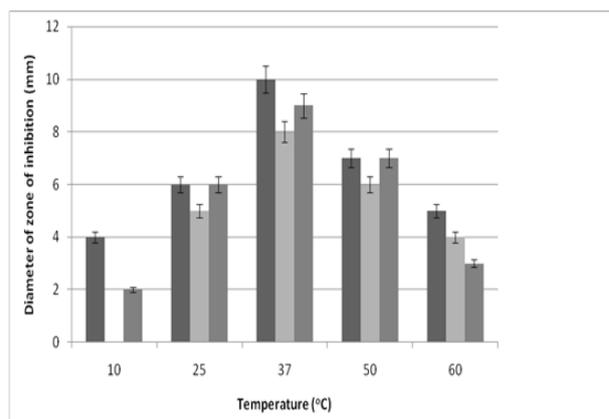


Figure 2. Effect of Temperature on antimicrobial activity of endotoxin of (■) *Salmonella typhi*; (■) *Salmonella typhimurium* and (■) *Shigella flexnerii*, on *Bacillus subtilis*.

## Conclusion

It should be noted that these are organisms which are mostly found in different food contaminated due to improper or unhygienic handling. The membrane protein molecules released after the cell lysis, are acted upon by the enzymes (usually serine proteases) like trypsin and then modified to form the toxins. However, all the cells do not undergo lysis. The other organisms in the intestine can overgrow or retard the growth of these organisms (especially *Shigella flexneri* and *Salmonella typhi*). Therefore, the bactericidal action of the toxin now comes handy as it serves as an auxiliary virulence factor to bring about the onset of the respective infection. It is also well known that proteins and lipids offer protection to different microorganisms (especially bacteria) against antimicrobial agents and the caseinase and lecithinase activity of the toxin molecules helps in removing this protection of other microorganism, thus enhancing their virulence. It also helps to bring about necrosis of the immunological barriers like the endothelial cells of the intestine and thus bring about a systemic infection.

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