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Factors Affecting Intra- and Extracellular Phospholipase A_1 Production by *Salmonella newport*

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ABSTRACT. The effect of various physico-chemical factors on production of intra- and extracellular phospholipase A_1 by *Salmonella newport* was investigated. Maximum intracellular enzyme levels were observed when cells were grown in brain heart infusion broth, after 12 h of incubation at 37 °C. Highest level of extracellular phospholipase A_1, however, was seen in synthetic medium (pH 7.0) after 24 h of incubation at 37 °C. Agitation during incubation had no effect on the intracellular enzyme synthesis but enhanced extracellular enzyme levels. Addition of surfactants to the growth media significantly decreased both intra- and extracellular phospholipase A_1 production.

The discovery of phospholipases, enzymes capable of phospholipid hydrolysis, in a host of biological objects ranging from cobra venom (Witcoff 1951) to bacteria (Avigad 1976) has opened up an interesting new area in the physiology and molecular biology of membranes. In recent years, a number of workers have reviewed the effect of bacterial phospholipases on cellular membranes, the toxic and pharmacological effects thereof and their usefulness as tools for membrane structure studies (Van den Bosch 1980; Waite 1985). The extensive work on potent phospholipase A_1 (EC 3.1.1.32) producer genera, such as *Escherichia coli* (Scandella and Kornberg 1971) and *Mycobacterium phlei* (Nishijima *et al.* 1974) has tended to ecclipse the investigations on other phospholipase-producing bacteria, such as salmonellas (Ames 1968). This resulted in a paucity of literature on phospholipases of these organisms. The present study was undertaken to examine the role of a number of physico-chemical factors on the synthesis of intra- and extracellular phospholipase A_1 by *Salmonella newport.*

MATERIALS AND METHODS

Bacterial strain. Salmonella newport strain 27656, an isolate from human faeces, was procured from the *National Salmonella and Escherichia Centre, CRI,* Kasauli (India) and used throughout the study. The culture was maintained on nutrient agar stabs (pH 7.2) at 4 °C and subcultured every three months.

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Effect of environmental factors. One mL of an 8-h-old culture in brain heart infusion broth (BHI) of the test organism was added to 100 mL of BHI in a 250-mL Erlenmeyer flask. The flasks were further incubated for 12 and 24 h at 37 °C on a gyrorotatory shaker (3 Hz). At the end of the incubation period, cells were harvested by centrifugation (8000 **g**, 20 min, 4 °C). The cell-free supernatant was collected and represented the extracellular phospholipase A_1 activity. The sedimented cells were washed twice with physiological saline, resuspended in 5 mL of Tris-HCl buffer (0.1 mol/L, pH 7.6) and sonicated (150 mA, 20 min) in an ice jacket. The sonicated material was centrifuged (5000 **g**, 20 min, 4 °C) and the supernatant was assayed for intracellular phospholipase activity. The growth at each stage was monitored by viable couting on MacConkey's agar.

Using the above basic strategy, the effect of a number of factors on phospholipase A_1 production was investigated.

Media: Enzyme production in BHI was compared with trypticase soy broth (TSB), nutrient broth, peptone water and synthetic media (Cruickshank 1970).

Temperature of incubation: The optimal temperature of incubation was determined by comparing the yields at 4, 28, 37 and 45 °C.

pH of media: The pH of BHI was adjusted to 3, 5, 7, 9, and 11 using HCl (0.1 mol/L) or NaOH (0.1 mol/L) and the enzyme production compared under standard conditions (37 °C, 24 h).

Aeration: Phospholipase A_1 production was studied in BHI incubated for 12 and 24 h at 37 °C under static and aerated conditions (3 Hz; gyrorotatory shaking).

Time of incubation: The organism was grown for 2, 4, 8, 12, 18, 24, 48 and 96 h at 37 °C in BHI.

Surfactants/antioxidants: Triton X-100, butylated hydroxyanisole (BHA), Lauricidin, Tween 80 and sodium dodecyl sulfate (SDS) were added at 0.1 % concentrations to BHI.

Estimation of phospholipase A_1 activity. Phospholipase A_1 levels were quantitated as per the method of Rao and Subramanyam (1969). Enzyme units (U) were expressed as μg of 1-lysophosphatidylcholine released per min per mg protein under standard conditions.

Protein estimation. Protein content of the enzyme was measured by the procedure of Lowry using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The nature of the growth medium had a significant effect on enzyme production (Table I). Maximum intracellular specific activity was seen in cells grown in BHI (5.0 U) which was significantly higher than in nutritionally poorer media, such as Davis and Mingioli's synthetic media ($p < 0.01$). Sooch (1982) has also indicated that *E. coli* cells grown in TSB and BHI had nearly 3.5 times more intracellular phospholipase A_1 activity than in the synthetic medium. The highest extracellular phospholipase A_1 production (201 U) was observed in the synthetic medium, nearly

TABLE I. Effect of growth media on growth (CFU/mL) and phospholipase A_1 production by *S. newport* after 24 h of incubation at 37 C

Growth medium	CFU/mL $\times 10^{-6}$	Phospholipase A_1 production*		
		Intracellular		Extracellular
		U/mg	U/mL	U/mg
Brain heart infusion	11 000	5.01 ± 0.73	45.2 ± 3.28	11.3 ± 0.82
Trypticase soy broth	42 000	4.82 ± 0.69[a]	41.7 ± 4.62[a]	9.9 ± 1.10[a]
Nutrient broth	9 000	2.60 ± 0.42[b]	11.7 ± 1.31[b]	6.5 ± 0.73[b]
Peptone water	4 800	4.92 ± 0.71[a]	21.3 ± 2.05[b]	9.3 ± 0.89[c]
Synthetic medium	300	2.85 ± 0.56[c]	4.2 ± 0.37[b]	201.4 ± 18.6[b]

*Statistical analysis of enzyme production in various media with respect to BHI.
[a] Change not significant.
[b] Significant at 1 % level ($p < 0.01$).
[c] Significant at 5 % level ($p < 0.05$).

TABLE II. Effect of incubation temperature on growth (CFU/mL) and phospholipase A_1 production by *S. newport* in BHI after 24 h of incubation

Temperature °C	CFU/mL $\times 10^{-6}$	Phospholipase A_1 activity, U/mg*	
		Intracellular	Extracellular
4	0.73	2.02 ± 0.52[b]	3.29 ± 0.41[c]
28	6500	3.25 ± 0.47[a]	6.98 ± 0.72[c]
37	72000	4.31 ± 0.64	11.25 ± 0.92
45	4.1	2.69 ± 0.56[b]	4.26 ± 0.58[c]

*Statistical evaluation of change in enzyme production at various growth temperatures with respect to 37 °C.
[a]Differences not significant ($p > 0.05$).
[b]Statistically significant at 5 % level.
[c]Statistically significant at 1 % level.

twenty-fold higher than in BHI ($p < 0.01$). This variation can be accounted for by the much higher protein content of the latter, since enzyme activity estimation per mL of broth revealed maximum phospholipase A_1 activity in cell-free supernatants from BHI (Table I). The presence of phospholipase A_1 activity in the synthetic medium, *i. e.* one without any phospholipids indicates that the production of the enzyme may be constitutive. The significant enhancement by ingredients of BHI merits further examination to assess the possible involvement of phospholipid--mediated stimulation of enzyme synthesis.

The optimum temperature for both growth and phospholipase A_1 production was 37 °C, followed by 28, 45 and 4 °C (Table II). The variations due to incubation temperature were more pronounced in extracellular than in intracellular phosoholipase A_1 levels. The initial pH of the medium had a strong influence on growth and enzyme production (Table III). Both enzymes were significantly higher at pH 7 than at the other pH values tested ($p < 0.01$). Bal (1983) has reported optimum pH for phospholipase A_1 production by *Salmonella* isolates to be 6.5, with no activity discernible at extreme pH values (3.5 or 9.5).

TABLE III. Effect of initial pH of medium on growth (CFU/mL) and phospholipase A_1 production by *S. newport* in BHI after 24 h of incubation at 37 °C

pH	CFU/mL $\times 10^{-6}$	Phospholipase A_1 activity, U/mg*	
		Intracellular	Extracellular
3 ± 0.5	—	—	—
5 ± 0.5	5 699	3.62 ± 0.51[b]	8.9 ± 0.96[a]
7 ± 0.5	73 000	6.05 ± 0.71	10.98 ± 0.84
9 ± 0.5	11 000	3.10 ± 0.63[b]	6.75 ± 0.73[b]
11 ± 0.5	0.16	—	1.32 ± 0.21[b]

*Statistical evaluation of change in enzyme production with respect to enzyme level at pH 7.0.
[a]Statistically significant at 5 % level.
[b]Statistically significant at 1 % level.

Aeration during incubation at 37 °C had no significant effect on intracellular phospholipase A_1 production (Table IV) but potentiated extracellular enzyme synthesis ($p < 0.05$). It remains to be ascertained whether the increase in extracellular phospholipase A_1 levels in aerated cultures is a consequence of enhanced release due to agitation or an oxygen-dependent effect.

TABLE IV. Effect of aeration on growth (CFU/mL) and phospholipase A_1 production by *S. newport* in BHI after 24 h of incubation at 37 °C

Growth condition	CFU/mL $\times 10^{-6}$	Phospholipase A_1 activity, U/mg*	
		Intracellular	Extracellular
Stationary	63 000	3.83 ± 0.61[a]	7.89 ± 0.74[b]
Shaker	80 000	4.25 ± 0.67	10.26 ± 0.91

*Statistical evaluation of change in enzyme level with respect to shake culture.
[a]Difference not significant ($p > 0.05$).
[b]Difference significant at 5 % level ($p < 0.05$).

Intra- and extracellular phospholipase A_1 synthesis increased with extended incubation period, reaching maximum values at 12 and 24 h, respectively, followed by a decline in enzyme activity. At 48 h, the intra- and extracellular phospholipase A_1 levels were only 15.8 and 42.0 % of the maximum activities (Fig. 1). Though intracellular phospholipase activity was detectable after only 4 h of incuba-

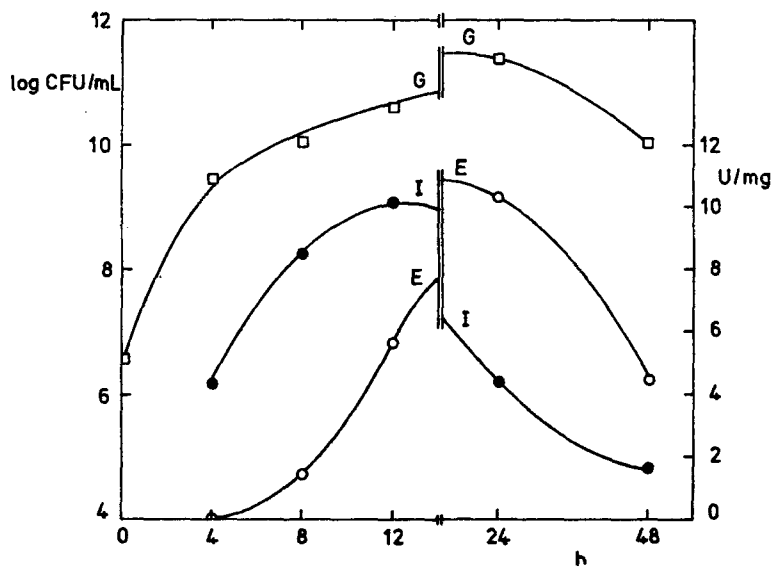

FIG. 1. Effect of time of incubation (h) on growth (G, log CFU/mL) and phospholipase A_1 production (U/mg); I – intracellular enzyme, E – extracellular enzyme.

tion, a minimum of 8 h growth was necessary for an observable extracellular enzyme activity. Sooch (1982) indicated no significant difference between phospholipase A_1 activity in *E. coli* cells harvested after 4 and 12 h of incubation at 37 °C. Bhandari and Asnani (1986), however, reported that highest intracellular phospholipase A_1 activity in *S. oranienberg* was seen in the mid-exponential growth phase, while optimum extracellular activity was detected in the late log or stationary stages of growth.

The addition of Lauricidin and SDS to BHI decreased the cell yield of *S. newport*. while Triton X-100, BHA, and Tween 80 did not appreciably alter the growth of the organism (Table V). Both intra- and extracellular phospholipase A_1 production were strongly inhibited by the addition of surfactants. With SDS, intra- and extracellular enzyme activity was only 70 and 57 % of the control values. Hashia *et al.* (1986) have also observed similar effects of decreased growth and phospholipase A_1 production in *E. coli* K_{12} by chemicals and biosurfactants.

In conclusion, it may be stated that both growth and phospholipase A_1 production by *S. newport* were strongly influenced by environmental and nutritional variables. The change in enzyme level, however, cannot be attributed to high/low viable counts alone since the changes in enzyme production were much higher (±50 %) while the cell count remained mostly within 1–2 log cycles (10^8–10^{10} CFU/mL).

TABLE V. Effect of surfactants on growth (CFU/mL) and phospholipase A_1 production by *S. newport* in BHI after 24 h of incubation at 37 °C

Surfactant	CFU/mL $\times 10^6$	Phospholipase A_1 activity, U/mg*	
		Intracellular	Extracellular
Control	83 000	4.25–0.69	10.26–0.83
Triton X-100	21 000	3.04–0.62[a]	5.90–0.71[c]
Lauricidin	9 600	3.85–0.51[a]	7.25–0.69[c]
Butylated hydroxyanisole	13 000	4.61–0.71[a]	6.21–0.81[c]
Tween-20	73 000	2.81–0.51[b]	6.98–0.62[c]
SDS	160	2.96–0.60[b]	5.85–0.69[c]

*Statistical evaluation of changes in enzyme level (with surfactant) with respect to control.
[a]Differences not statistically significant ($p > 0.05$).
[b]Significant at 5 % level ($p < 0.05$).
[c]Significant at 1 % level ($p < 0.01$).

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