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Cellular Location of Streptolysin O

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Streptolysin O was measured in subcellular fractions of group A streptococci obtained after preparation of protoplasts in a hypertonic buffer containing raffinose. Most of the activity was located in the periplasm (the region between cell wall and membrane) and did not differ in several characteristics from that of extracellular streptolysin O. Of the enzymes used as subcellular markers, aldolase and maltase (cytoplasmic) and acid phosphatase (membrane associated) were in the same fractions as found in other bacteria. However, the location of alkaline phosphatase differed from that of other bacteria in that most of the activity was in cytoplasm rather than in the periplasm.

The presence of cellular streptolysin O (SLO) has been documented previously (22), but little is known about its subcellular location. Although production of hemolysin (possibly SLO) by organisms presumed to be protoplasts (12) has been noted, the presence of intracellular pools of SLO has not been documented. Studies wherein streptococci were lysed by grinding or sonic treatment have indicated that easily measurable levels of cell-associated SLO are present (22) but have not been useful to answer the question of whether SLO is mesosomal, periplasmic, membranous, or intracellular in location. The study reported here documents the periplasm as the major location for SLO.

MATERIALS AND METHODS

Strains and growth conditions. The strains listed below were from the Laboratory of Streptococcal Diseases (LSD) collection. Group A streptococcus strain C203S (LSD 73x45) originally received from Alan Bernheimer, a type 49 Red Lake strain (LSD 60x311), and a type 1 strain (LSD CB51-4504) were used in these experiments. Each strain was initially grown in brain heart infusion broth (BBL) and then resuspended in heart infusion broth (Difco) with 0.3% added maltose (HI-3% maltose) for final growth. Cells were harvested during mid to late log phase (75 Klett-Summerson units, no. 520 filter, unless otherwise indicated), washed once with 0.85% saline, and suspended in either hypotonic buffer (0.03 M sodium phosphate, pH 6.5; 0.85% NaCl; and 10^{-3} M 2-mercaptoethanol) or hypertonic buffer (hypotonic buffer plus 30% raffinose and 10^{-1} M $MgCl_2$).

PAL preparation. Phage-associated lysin (PAL) was prepared and titered as described previously (6).

Preparation of protoplasts and subcellular fractions. Protoplasts were prepared by incubation of streptococci in 10 ml of hypertonic buffer at 37 C for 40 min with PAL of such titer that less than 0.2 ml could lyse all cells from 1 liter of culture in hypotonic buffer. Protoplasts were then sedimented by centrifugation at 30,000 × *g* for 40 min. The mesosomal fraction was sedimented from the supernatant fluid by centrifugation for 2 h at 120,000 × *g* and washed once with 0.85% saline. The remaining supernatant fluid was designated as the periplasmic fraction. The protoplasts were lysed in hypotonic buffer without 2-mercaptoethanol and then incubated with deoxyribonuclease (1 mg/ml) for 15 min at 37 C. Debris was then removed by centrifugation of the mixture at 4,000 × *g* for 10 min. The membranes were separated from the cytoplasm by centrifugation at 30,000 × *g* for 40 min and then washed two times with 0.85% saline.

Hemolysin assay. The assay procedure was a modification of that used by Schwab (21). Sheep erythrocytes, freshly supplied commercially in Alsever solution, were centrifuged at 3,600 × *g* for 10 min, and the supernatant and buffy coat were discarded. The erythrocytes were suspended in 0.8% NaCl in 0.03 M sodium phosphate, pH 6.5. One unit of hemolysin (1 HU) produced 50% lysis of a 0.3% erythrocyte suspension in 75 min at 37 C.

Inhibitors of SLO. Antistreptolysin O serum (BBL) (various amounts) or *N*-ethelmaleimide (5 to 20 mM) was mixed with SLO and held at 0 C for 10 min, and the mixture was assayed for hemolytic activity.

Enzyme assays. Aldolase was assayed using the aldolase Stat-Pack (Calbiochem) and reported as change in optical density at 340 nm per minute/milligram of protein. Alkaline and acid phosphatase were assayed by the procedure of Reaveley and Rogers (20), and reported as micromoles of *p*-nitrophenol released/milligram of protein. Maltase was assayed by the procedure of Doolin and Panos (11). Protein was measured by the method of Lowry et al. (15).

Sephadex G-100 chromatography. SLO was chromatographed on a 40-cm Sephadex G 100 column (2.5 by 40 cm) eluted with hypotonic buffer.

RESULTS

When group A streptococcus strain C203S was grown in HI-0.3% maltose, the highest ti-

ter of cell-associated SLO occurred during mid to late log phase of growth (Fig. 1), also a period of maximal extracellular accumulation (data not shown). Slightly lower titers were obtained when cells were disrupted with PAL at 37 C instead of 23 C, but the relationship of SLO titer to cell growth remained the same.

The cell-associated SLO hemolysin exhibited several of the same characteristics as extracellular SLO; that is, it was inhibited by cholesterol anti-streptolysin O serum (BBL) (1), and *N*-ethyl maleimide, a sulfhydryl inhibitor (19, 23). The onset of hemolysis by cellular SLO was immediate, unlike that for streptolysin S (SLS) (4). Finally, the titer was unaffected by incubation with ribonucleic acid core which increases the titer of SLS (5, 13).

Criteria used to monitor the stability of protoplasts prior to the preparation of subcellular fractions to localize SLO were osmotic fragility and enzyme markers. Osmotic stability of the PAL-treated cells was monitored by dilution of the mixtures into the original hypertonic buffer and into water. After 40 min, protoplasts were fragile in water, losing 83% of their original turbidity, but were relatively stable in hypertonic buffer, losing an average 23% of their original turbidity. Various enzymes were assessed as subcellular markers during the protoplast procedures. The cytoplasm always contained the largest amounts of aldolase, some 11 to 51 times as much as in the periplasm. Most of the maltase, as previously found by Doolin and Panos in L forms (11), was also cytoplasmic in location. The distribution of acid and alkaline phosphatase in a type 49 Red Lake strain (results similar for strain C203S) is shown in Table 1. Acid phosphatase, as found in other organisms (18, 20), was predominately in the membrane fraction. However, the alkaline phosphatase which has been associated with the periplasmic fraction in other organisms (8, 10, 18) was predominately cytoplasmic in location.

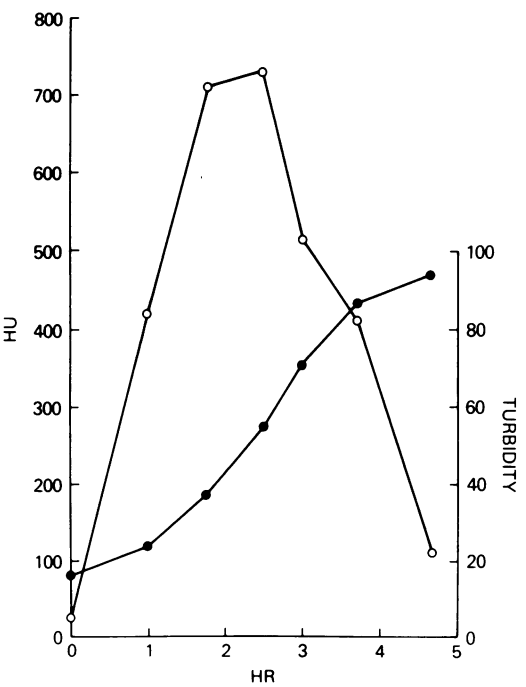

FIG. 1. *Relationship of cellular SLO to cell growth. Strain C203S cells were resuspended in 5 ml of hypotonic buffer and incubated with PAL for 20 min at 23 C. The supernatant, after centrifugation of the lysate at 20,000 × g for 15 min, was assayed for SLO. After correction of the titer to a common cell mass (titer/optical density), the activity was reported as HU/ml (HU)* (○). *Turbidity* (●) *was measured with a no. 520 filter (Klett-Summerson).*

The titer of SLO in subcellular fractions was determined in mid- to late-log phase cells incubated with PAL in the hypertonic buffer indicated above. Most of the SLO activity in strain C203S is localized in the periplasm, in a ratio of more than 10:1 to that in the cytoplasm (Table 2). The amount of SLO secreted by cells in hypotonic and hypertonic buffer was also measured, since the titer in the periplasm may, in part, be due to SLO secreted by cells during the preparation of protoplasts. Cells in hypertonic buffer secreted less than 10% of that measured in the periplasm. The probable reason for the low titer of SLO secreted by these cells was the presence of 30% raffinose. Use of lesser amounts of raffinose or even sucrose (15 and 30%) resulted in the same findings, suggesting that secretion of SLO was prevented by hypertonicity. Interestingly, when these cells were resuspended in hypotonic buffer, they did not secrete more than trace amounts of hemolysin. In other strains of group A streptococci, such as a type 49 Red Lake and a type 1 (a strain isolated from a rheumatic fever patient), SLO was also predominantly in the periplasm fraction.

Investigations of extracellular SLO by other investigators (2) has revealed a dimeric as well as a monomeric form of SLO. We, like Taketo and Taketo who disrupted cells by sonic treatment (22), found only one major peak of SLO activity (approximately 34 ml after the void volume) after chromatography of a cell lysate on Sephadex G-100. The molecular weight of 53,000 determined by the method of Andrews (3) is in approximate agreement with that of others for the monomeric form (2). The presence of low-titer hemolysin measurable in the eluate

TABLE 1. *Localization of cellular enzymes in group A streptococcus (type 49 Red Lake)*

Enzyme	Sp act[a]				Total act[b]			
	cyto[c]	peri	mem	meso	cyto	peri	mem	meso
Acid phosphatase	0.22	2.6	8	25	18	42	240	30
Alkaline phosphatase	0.24	0.17	0.21	2.2	20	2.8	6.3	2.6
Aldolase	2.6	0.5	ND[d]	ND	205	8	ND	ND

[a] See Materials and Methods for units.
[b] Fractions prepared from cells from a 0.9-liter culture.
[c] cyto, Cytoplasm; peri, periplasm; mem, membrane; meso, mesosome.
[d] ND, None detected.

TABLE 2. *Subcellular location of SLO in group A streptococcus strain C203S*

Location	Volume (ml)	Protein (mg/ml)	SLO (HU/ml)	SLO (Total HU)	SLO (HU/mg of protein)
Cytoplasm[a]	5.6	2.15	42	235	19.5
Periplasm	6	1.5	428	2,568	285
Mesosome	2	0.53	17	34	64
Membrane	5	1.05	13	65	12
Hypotonic supernatant[b]	6	ND[c]	300	1,800	ND
Hypertonic supernatant[b]	6	ND	33	200	ND

[a] Fractions prepared from cells from a 0.2-liter culture.
[b] Cells were incubated without PAL for 40 min in hypertonic or hypotonic buffer, and the suspension was then centrifuged at 10,000 × *g* for 20 min. The supernatant was assayed for SLO activity.
[c] ND, Not done.

just after the void volume may have represented aggregated forms of SLO.

DISCUSSION

Only a few studies on bacterial hemolysins have been concerned with subcellular localization (9, 16). Perhaps the major reasons have been the difficulty in preparation of protoplasts of the appropriate organisms for successful subcellular localization or in prevention of cytoplasmic leakage from presumed protoplasts. For example, the lack of a suitable procedure to prepare protoplasts has made the subcellular localization of any hemolysin in group B streptococci impossible. We have had great difficulty preparing stable protoplasts of group A streptococci when using buffers containing sucrose or NaCl as osmotic stabilizing agents. The presence of raffinose and high Mg^{2+} concentration, as used previously in this lab by A. Schade (unpublished observations), has prevented cytoplasmic leakage or premature rupture of streptococcal protoplasts. This procedure has also been applied to the successful preparation of protoplasts of group H streptococci (6).

Several degradative enzymes which accumulate in the extracellular medium have been localized in the periplasm of microorganisms (17, 18). However, the periplasmic location for a bacterial hemolysin like SLO has apparently not been reported before. Whether this is only a transitory location for SLO is at present unknown. Although a periplasmic location of other hemolysins is probably predictable (work in progress suggests that this, in addition to other locations [9, 16], is true for staphylococcal α-toxin), this is not true of all hemolysins. For example, the major subcellular location of a recently isolated cellular SLS-related latent hemolysin (7) is not the periplasmic space (unpublished observations).

By the methods employed, no major differences between the extracellular and cellular form of SLO have been found. There is no reason to suggest that activatable forms are present as for other streptococcal products such as proteinase (14) and SLS (7).

The observation that the major cell location of alkaline phosphatase is cytoplasmic is interesting since the location in all other organisms is periplasmic. It does not appear that the presence of alkaline phosphatase in the cytoplasm is artifactual, but alternative cytochemical methods to localize alkaline phosphatase are in progress to further document this observation.

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