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Effect of Detergents on Streptolysin S Precursor

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Group A streptococci which produce streptolysin S contain a cellular precursor to streptolysin S in the membranes and cytoplasm which is activatable by blending in a Vortex mixer with glass beads and ribonucleic acid (RNA)-core (RNA preparation from yeast). Although no activation of precursor occurred when it was mixed with detergents, it was activated when blended with glass beads and detergents such as Tergitol NP-40 and Brij 35. Maximum activation of precursor was achieved in 1 to 2% detergent, in pH 6.5 buffer, and after 8 min of blending. Detergents Tween 20, 40, 60, and 80, Brij 56, and Lubrol WX also activated precursor, but, of all the hemolysin preparations, those with Tween 40 or 60 or Lubrol WX were the most stable. The addition of RNA-core during or after blending of precursor with detergents enhanced the titer and stability of the hemolysin. This was due in part to the association of the hemolytic moiety with RNA-core. Activation of precursor in the membrane was better with a detergent, whereas that in the cytoplasm was better with RNA-core. Therefore, precursor from two different cellular locations can be differentiated by the effects of RNA-core and detergents on precursor titer.

Group A streptococci which produce extracellular streptolysin S (SLS) contain a cellular SLS precursor (2). The mechanism by which active hemolysin is formed in vivo from the precursor is unknown but appears to be an energy-dependent process (4) as found for SLS formation by resting cells (1). Ginsburg and Grossowicz (6) noted the formation of an SLS-like hemolysin when cells were incubated with the detergent Tween 40, and they suggested that a cell-bound hemolysin had been released. SLS precursor can be detected in cell membranes, mesosomes, and cytoplasm (unpublished observations). Since detergents may solubilize membrane proteins (8, 11, 12), their effects on SLS precursor in isolated membranes are of interest in terms of any relationship of precursor to the previously described cell-bound hemolysin (6), the possibility of removing precursor from the membranes, and any differences in effect on precursor from membrane as compared with that from cytoplasm. This paper reports the activation of SLS precursor by several detergents by blending in a Vortex mixer with glass beads and the differentiation of precursor in membranes from that in the cytoplasm.

MATERIALS AND METHODS

Membrane and cytoplasmic SLS precursor. Fractions containing precursor were prepared from group A streptococcal strains CB11 2252 or 2798 (LSD 78x4) (originally obtained from H. Dillon, University of Alabama) which were grown to late log phase in heart infusion broth-0.3% maltose as previously described (2). Protoplasts of 78x4 and CB11 2252 were prepared as previously described (3), and membranes were separated from the cytoplasm by centrifugation (20,000 × *g* for 60 min) of the protoplast lysate. Membranes from CB11 2252 were also isolated from late-log-phase cells lysed with mutanolysin (4 mg per cells from 1 liter of culture) (13) (generously supplied by K. Yokogawa, Dianippon Pharmaceutical Co. Ltd., Osaka, Japan) in buffer (0.03 M KPO_4, pH 6.5, plus 0.85% NaCl) at 37°C for 60 min; the membranes were sedimented at 20,000 × *g* for 30 min at 4°C.

Activation of precursor. Samples of membranes containing SLS precursor from CB11 2252 (0.5 mg of protein in all experiments except as described in footnote *b* in Table 6) or 78x4 (0.8 mg of protein) to be activated were mixed with ribonucleic acid (RNA)-core (RNA preparation from yeast) or detergent and buffer to 2 ml of total volume and 3 ml of washed glass beads in a screw-cap tube (16 by 125 mm). The tube was blended for 15 min at a 45° angle on a Vortex Genie with intermittent cooling in ice. The sample was removed after addition of 2 ml of buffer and sedimentation of the beads.

Glass beads. MX-XLX glass beads were obtained from Cataphote-Ferro Corp., Jackson, Miss. They were washed several times with 3 to 4 volumes of deionized water; any metal fragments were removed with magnetic stirrers, and washing was continued with 1 to 2 volumes of 25% and then 60% acetic acid. Beads were again washed several times in deionized water and finally in 0.03 M $NaPO_4$, pH 6.5.

Detergents. The following detergents and chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.: Tween 20, 40, 60, and 80; Tergitol TMN, XD, 15S9, NP-14, and NP-40; Brij 35 and 56; Triton

X-100; Digitonin; hexadecyltrimethyl ammonium bromide; ethylene glycol monomethyl ether; and Lubrol WX.

RNA-core. RNA-core was prepared as described previously (2).

Protein determinations. Protein content was determined by the method of Lowry et al. (10).

Hemolysin assay. The hemolytic activity was measured with sheep erythrocytes as described previously (2). The stability of the hemolysin preparation was measured at 18, 24, or 48 h after storage of the samples in an ice bath.

Ethanol precipitation. Absolute ethanol (3 volumes) at −20°C was added to hemolysin preparations at 0°C. The mixtures were kept at 0°C for 5 min and then centrifuged at 10,000 × *g* for 20 min at −20°C. The precipitate was suspended and assayed for hemolytic activity.

Chromatography on Sepharose 6B. Hemolysin (2,500 hemolytic units [HU]) formed by grinding precursor with Tergitol NP-40 and RNA-core was chromatographed on a column (2.5 by 65 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) with an elution buffer consisting of 0.03 M KPO_4 (pH 6.5) and 0.85% NaCl.

RESULTS

No activation of precursor occurred when membranes were suspended in Tween 80 (1%), Tergitol NP-40 (1%), Brij 35 (1%), or phosphate buffer for 30 min at room temperature. (Although Brij 25 at low dilutions caused lysis of erythrocytes, there was no hemolysis when the Brij 35-membrane suspension was added to erythrocytes.) When membranes exposed to Tergitol NP-40 were subsequently sedimented and assayed for precursor by grinding with glass beads and RNA-core, there was no loss of activity. In fact, the titer was slightly higher than that of the control. Therefore, the effect of blending membrane with detergent and glass beads was examined. It was found that precursor in membranes from group A streptococcal strains CB11 2252 and 78x4 was activated by grinding in the presence of the detergent in the absence of an added carrier (Table 1). The titer was higher with Tergitol NP-40 than with RNA-core, but it was highest when both were present. This was also true when Brij 35 was substituted for Tergitol NP-40. (The hemolysin was assayed at dilutions at which the residual Brij 35 in the mixture would not cause hemolysis.) The optimum concentration of Tergitol NP-40 for activation of precursor was 1 to 2%; that is, for strain 78x4, 175, 500, 550, and 575 HU/ml were formed with 0.1, 0.5, 1, and 2%, respectively. Maximal activation occurred by 8 min (Table 2). The titer was not adversely affected, however, by more prolonged blending, at least up to 15 min. The titer was highest at pH 6 when precursor was blended with Tergitol NP-40, but was highest at pH 6.5 when Brij 35 was used (Table 3). When the activated precursor-detergent preparation was centrifuged at 100,000 × *g* for 60 min, all activity remained in the supernatant, demonstrating that activated precursor had been solubilized from the membrane.

A major problem was the instability of the activated hemolysin. At 0°C, 20% of the activity of the hemolysin in Brij 35 or Tergitol NP-40 was lost in 3 h, and more than 50% was lost by 18 h. The addition during blending of Mg (10^{-2} or 10^{-3} M) or an increase in the concentration of phosphate (to 0.1 M) had no effect on titer or stability, whereas addition of ethylenediaminetetraacetic acid (10^{-2} M) resulted in a titer 2.5

TABLE 1. *Effect of RNA-core and Tergitol NP-40 to activate membrane SLS precursor from strains CB11 2252 and 78x4*

Addition[a]	HU/ml	
	CB11 2252	78x4
Nothing	15	0
RNA-core	1,100	430
Tergitol NP-40 plus RNA-core	2,000	750
Tergitol NP-40	1,200	580

[a] RNA-core (2 mg) or Tergitol NP-40 (1%) was added to membrane precursor, the volume was adjusted to 2 ml with buffer, and the mixture was ground with glass beads.

TABLE 2. *Effect of time of blending with Tergitol NP-40 on activation of membrane SLS precursor of CB11 2252*[a]

Time (min)	HU/ml	Activation (%)
2	355	27
4	690	53
6	800	62
8	1,300	100
10	1,200	90
15	1,300	100

[a] Procedure as in Table 1.

TABLE 3. *Effect of pH on activation of CB11 2252 membrane SLS precursor by Tergitol NP-40 and Brij 35*[a]

pH[b]	HU/ml	
	Tergitol NP-40	Brij 35
6.0	1,500	1,600
6.5	1,400	1,800
7.5	1,300	1,400
8.0	500	250

[a] Detergent (1%) was added to membrane precursor, the volume was adjusted to 2 ml with buffer, and the mixture was ground for 15 min with glass beads.

[b] KPO_4 (0.03 M) plus 0.85% NaCl.

times lower. After activation of precursor in Tergitol NP-40 or Brij 35, neither changing the pH (6.0, 6.5, 7.5, or 8.0) nor changing the detergent concentration (0.5, 1.0, 1.5, or 2.0%) enhanced the stability of the active hemolysin.

A comparison of the abilities of various detergents to activate precursor in membrane to determine which might be best was made at a concentration of detergent and at pH 6.5. Triton X-100, Digitonin, hexadecyltrimethyl ammonium bromide, ethylene glycol monomethyl ether, and Tergitol TMN, XD, 15S9, and NP-14 were all ineffective. However, several other detergents were useful (Table 4), and their use resulted in higher-titer hemolysin than that obtained with RNA-core. Tween 20, 40, 60, and 80 and Tergitol NP-40 alone did not cause hemolysis, but Brij 35 and 56 and Lubrol WX did. The assays for the hemolysins obtained with these last three detergents were done at dilutions at which the residual detergent caused no hemolysis. Although use of Tween 20, 40, 60, and 80 yielded equal degrees of activation, only in Tween 40 and 60 did hemolysin retain a significant portion of its activity for 48 h in an ice bath. Hemolysin in Lubrol WX was also of high titer and retained most of its activity for the same time. However, after 48 h, the residual titers of Tween 40 SLS, Tween 60 SLS, Lubrol WX SLS, and RNA-core SLS were about the same.

RNA-core, when added to precursor and detergent during the activation, enhanced not only the titer but usually the stability (Table 5). This effect was also noted when RNA-core was mixed with the hemolysin-detergent mixture at 37°C (Table 5) or 0°C (data not shown). This suggested that the hemolytic moiety of the detergent hemolysin probably bound to the RNA-core, forming the stable RNA-core SLS (5), analogous to the transfer of the hemolytic moiety of Tween 40 SLS formed extracellularly to RNA-core (7). The effect of ethanol, which precipitates RNA-core, on the different hemolysins was consistent with this. That is, 75% ethanol at 0 to −20°C precipitated all of the hemolytic activity of RNA-core SLS or the hemolysin formed with Tergitol NP-40 and RNA-core, but none of the activity of hemolysin formed with Tergitol NP-40 or Tween 40. When the hemolysin formed with Tergitol NP-40 and RNA-core was chromatographed on Sepharose 6B, it had the same elution volume as RNA-core SLS (data not shown).

Some precursor is present in the cytoplasmic fraction of the streptococci (unpublished observation), but as yet its relationship to that in the membrane is uncertain. Precursor in the cytoplasm is sedimentable by high-speed centrifugation, and it is therefore possible that such precursor could be associated with membrane fragments. The effect of detergent to activate cytoplasmic material was, therefore, of interest. When the titer of membrane precursor obtained with Brij 35 was compared with that of RNA-core, the ratio was always greater than 1 (Table 6). However, the ratio in the cytoplasm was always 1 or less. The titer of cytoplasmic precursor which was sedimented was two or more times higher with RNA-core than with Brij 35. The hemolysin titers for cytoplasm sediment 3 and residual cytoplasmic supernatant were about the same as those for Brij 35 at this dilution. However, similar results for these prep-

TABLE 4. *Activation of CB11 2252 SLS membrane precursor by detergents and RNA-core and the stability of the hemolysin*[a]

Detergent	HU/ml	% of titer remaining	
		24 h	48 h
Tween 20	1,900	0	—[b]
Tween 40	1,900	58	57
Tween 60	1,900	86	54
Tween 80	1,850	17	—
Tergitol NP-40	1,240	42	23
Brij 35	1,640	38	21
Brij 56	2,340	21	—
Lubrol WX	1,900	68	68
RNA-core	950	100	126

[a] Procedure as in Table 3.
[b] —, Not done.

TABLE 5. *Effect on detergent hemolysin of RNA-core added during (A) or after (B) the activation of membrane SLS precursor from CB11 2252*

Addition[a]	HU/ml	
	A[b]	B[c]
RNA-core	1,070 (1,260)[d]	—[e]
Tergitol NP-40	1,220 (480)	2,200 (2,240)
Tergitol NP-40 plus RNA-core	2,200 (2,640)	—
Tween 40	1,600 (1,330)	1,980 (1,660)
Tween 40 plus RNA-core	1,840 (1,740)	—
Lubrol	1,600 (1,250)	2,040 (1,840)
Lubrol plus RNA-core	1,600 (1,900)	—

[a] RNA-core (2 mg), detergent (1%), or both were added to membrane precursor, the volume was adjusted to 2 ml with buffer, and the mixture was blended with glass beads.
[b] Titer immediately after blending with glass beads.
[c] Titer after RNA-core (2 mg) was added to 1 ml of detergent hemolysin and the mixture was incubated at 37°C for 15 min.
[d] Numbers within parentheses are titers after mixtures were held for 18 h in an ice bath.
[e] —, Not done.

TABLE 6. *Activation of CB11 2252 SLS precursor in membrane[a] and cytoplasm[b] by RNA-core and Brij 35*

Precursor fraction	HU/ml		Brij 35 SLS/RNA-core SLS ratio
	Brij 35 SLS	RNA-core SLS	
Membrane	700	350	2
Cytoplasm	1,200	1,200	1
Cytoplasm sediment 1	210	450	0.5
Cytoplasm sediment 2	2,400	4,500	0.5
Cytoplasm sediment 3	42	440	0.1
Cytoplasm supernatant	45	590	0.1

[a] Membranes from protoplasts were washed twice with buffer and stored frozen. A 0.1-ml sample (0.2 mg of protein) was blended for 15 min with glass beads, RNA-core (2 mg) or Brij 25 (1%), and buffer to adjust the volume to 2 ml.

[b] A sample (8 ml) of cytoplasm from protoplasts or the resultant cytoplasm supernatant was centrifuged sequentially at 50,000 × *g* for 1 h, 100,000 × *g* for 2 h, and 120,000 × *g* for 4 h. The three sediments were each suspended in 3 ml of buffer. Sediments 1, 2, and 3 were obtained at low-, intermediate-, and high-speed centrifugation, respectively. A sample (0.5 ml) of cytoplasm sediment 1 (0.03 mg of protein), sediment 2 (0.6 mg of protein), sediment 3 (0.06 mg of protein), cytoplasm (2 mg of protein), or cytoplasm supernatant (1.4 mg of protein) was blended for 15 min with glass beads, RNA-core (2 mg) or Brij 35 (1%), and buffer to adjust the volume to 2 ml.

arations were obtained with Tween 40. There was, therefore, a very distinct difference between precursor in the membrane and the cytoplasm.

DISCUSSION

SLS precursor was activated by grinding with glass beads in the presence of any of several detergents. The addition of a known carrier such as RNA-core (1) was not necessary for the activation, but it potentiated the titer and enhanced the stability. The change in titer and stability is due to the binding of the hemolytic moiety to RNA-core and the formation of the stable RNA-core SLS (5). Such a reaction occurs when the hemolysin formed with Tween 40 (Tween SLS) in the resting cell system is mixed with RNA-core (7). The resultant hemolysin has all the properties of RNA-core SLS (5).

Ginsburg and Grossowicz (6) speculated that Tween 40 or detergents released a cell-bound hemolysin that was related to SLS. It is unlikely that extraction is the mechanism for the formation of Tween SLS since mixing of precursor with detergent resulted in no activation. Ginsburg and Grossowicz (6) have shown that metabolic inhibitors markedly decrease the formation of Tween SLS. It is more likely that the detergent stabilized a secreted peptide (5, 9). As suggested by Ginsburg and Harris (7), Tween SLS and RNA-core SLS probably have the same hemolytic moiety. Both hemolysins are formed from the same precursor, which does indeed appear to be cell bound.

Precursors in the membrane and the cytoplasm are distinguishable by activation by RNA-core or detergents. The titer of precursor in membrane is always higher than RNA-core when Tergitol NP-40, Brij, Tween, or Lubrol WX is used. However, activation of cytoplasmic precursor is better when RNA-core is used rather than a detergent. Whether this differential effect is due to lability of the cytoplasmic precursor in the presence of the detergent or to the presence of cofactor in the membrane preparation is unknown.

Others have found detergents useful for solubilization of membrane components such as enzymes, but the degree of solubilization is often inversely related to the activity of a solubilized enzyme (8). No activation occurred when precursor was mixed with detergent; therefore, solubilization presumably occurred only during the blending procedure. The results in this study differ from those on the solubilization of membrane components such as adenylate cyclase (8) in that the activity of hemolysin was not related to the hydrophilic lipophilic balance number of the detergent as was true for adenylate cyclase. Presumably the use of glass beads with detergent compared with detergent alone played a role in this. Although the Tweens solubilized little of the adenylate cyclase (8), they were very effective with glass beads in this system. Why the difference between 14-, 16-, 18-, and 20-carbon lengths in the fatty acids of the Tweens should make so much difference in the stability of SLS is not known. The detergent hemolysin could be the hemolytic moiety with or without a membrane lipid in a complex with detergent. If the character of such a hemolysin is to be examined, Tween 40 or 60 or Lubrol WX appears to be the best detergent to use.

The activation of precursor by detergents may be of use in the purification of the hemolytic moiety of SLS. Marked increases in the yield of RNA-core SLS from precursor can be obtained with the detergent. RNA-core SLS can also be prepared from the detergent hemolysin; this could potentially decrease nonspecific binding of cell components to RNA-core as might occur during blending of precursor with RNA-core. The potential benefit of these procedures remains to be determined.

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