The Role of NaCl in the Lysis of *Staphylococcus aureus* by Lysostaphin

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SUMMARY

Lysostaphin attacked both viable staphylococci and the mucopeptide portion of the staphylococcal cell wall. In the absence of salts, lysostaphin activity could only be recovered from the particulate portion of the lysed cell after centrifugation, whereas in the absence of salts its action on the mucopeptide resulted in a recovery of active material in both the sediment and the supernatant fluid. It appears from these observations that lysostaphin is complexed with its substrate and that NaCl is required to break the complex.

INTRODUCTION

Schindler & Schuhardt (1964) reported on the isolation of an organism of the genus *Staphylococcus* that produced a lytic agent, lysostaphin, active against all of 59 strains of staphylococci tested. The specificity for organisms of the genus *Staphylococcus* separated its mode of action from that of lysozyme. The authors also described an assay for determining the quantity of enzyme based upon the decrease in turbidity of a standard staphylococcal strain. Since the rate of lysis of a staphylococcal strain was a function of the concentration of lysostaphin present, it was possible to deviate from the standard assay micro-organism in these experimental procedures.

Schindler & Schuhardt (1965) purified and described some of the properties of lysostaphin and reported that it was a basic protein essentially devoid of cystine and cysteine and contained large quantities of aspartic and glutamic acid compared to the total amino acid content. The lytic protein had a sedimentation coefficient ($S_{20,w}$) of 2·35S and an isoelectric point between pH 10·4–11·4. The rate of lysis of *Staphylococcus aureus* by lysostaphin was dependent upon the concentration of NaCl present in the medium. This phenomenon paralleled the salt requirement of the muramidase lysozyme (Salton, 1957). By increasing the concentration of NaCl in the suspending medium from zero to 0·15 M, an increased rate of lysis of staphylococci was observed. Concentrations of salt above physiological values introduced parameters which affected both the bacterial cells and the stability of lysostaphin; thus such levels of salts were not evaluated. This investigation was undertaken to determine in what manner NaCl entered into the dynamics of the lysostaphin-substrate interaction.

METHODS

Micro-organism. *Staphylococcus aureus*, strain sv, the mouse-virulent variant of the Smith strain (Hunt & Moses, 1958) was grown in Trypticase soy broth at 37° under conditions of aeration and agitation to give a maximum yield of cocci/ml.

The cocci were harvested by centrifuging at 8000*g* for 15 min. and washing with 0.05 M-tris buffer, pH 7.5.

Preparation of cell walls. Sixteen g (wet weight) of washed *Staphylococcus aureus* (sv) were suspended in 1 M-NaCl and mechanically ruptured in a Nossil disintegrator (McDonald Engineering Co., Bay Village, Ohio) with no. 13 Ballotini beads. The equipment was operated for six 1 min. periods with the capsule kept under a constant flow of liquid CO₂ to minimize autolysin action. The suspension was passed through a coarse sintered glass filter to remove the glass beads and the walls were recovered by centrifugation as described by Perkins & Rogers (1959). The cell walls were treated with the enzymes trypsin, ribonuclease and pepsin according to the procedure of Morse (1962). Residual protein and lipid were chemically extracted by the method of Mandelstam (1962). The yield after drying *in vacuo* was 300 mg. The homogeneity of the preparation was checked in a Siemens Elmiskop electron microscope with a beam magnification of $\times 10,664$.

Chemical analysis of cell wall. Total phosphorus was determined by the method of Fiske & SubbaRow (1925), and ribitol by thin-layer chromatography utilizing cellulose (Camag type D, Arthur Thomas, Phila., Pa.) at a thickness of 250 μ as the support and developed with *tent*-butanol + acetic acid + water (52 + 3 + 25, by vol.). The plates were sprayed with acetylacetone-*p*-dimethylaminobenzaldehyde reagent (Dawson, Elliot, Elliot & Jones, 1959) and observed for fluorescence under ultraviolet radiation (2570 Å).

N-terminal amino acids were determined by combining the treated cell walls under alkaline conditions with absolute ethanol containing 2,4-dinitrofluorobenzene. The mixture was placed on a shaker for 4 hr at room temperature. Excess 2,4-dinitrofluorobenzene was removed by continuous extraction with absolute ether until no further colour could be observed in the ether phase. The aqueous phase was brought to dryness and hydrolysed *in vacuo* with 6 N-HCl for 22 hr at 110°. The HCl was removed by drying *in vacuo* at 45° over KOH pellets and the presence of the coloured dinitrophenyl derivatives was determined by thin layer chromatography with Silica Gel G (Research Specialties Co., Richmond, Calif.) at a thickness of 150 μ as the support and developed with *n*-butanol + methyl ethyl ketone + water (2 + 2 + 1, by vol.). The plates were photographed through a 47B filter on Kodalith film (Eastman Kodak Co., Rochester, N.Y.).

Total amino acids and amino sugars were determined on a Technicon Analyzer (Technicon Corp., Chauncey, New York) according to the instructions of the manufacturer.

Preparation of enzyme and determination of its activity. Lysostaphin prepared and purified by Mead Johnson Research Center, Evansville, Indiana, was dialysed for 24 hr against 1000 times its volume of tris buffer, pH 7.5, with frequent changes of the dialysing fluid. Samples of this material were assayed at 37° against a washed suspension of *Staphylococcus aureus* (sv) with NaCl added to give a 0.15 M concentration. Turbidimetric readings were recorded initially and at 5 min. intervals utilizing a Coleman Jr. Spectrophotometer operated at a wavelength of 540 μ . An equivalent sample of lysostaphin was added to a similar suspension of *S. aureus* with the exception that no NaCl was present. Turbidimetric readings were made as above; after 15 min. incubation the suspension was centrifuged in a Spinco Model L Preparative Centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.)

at 33,000*g* for 15 min. at 4° and the sediment washed 3 times with tris buffer. The combined supernatant fluids from the washings were assayed for lytic activity against a suspension of *S. aureus* similar to that used in the initial enzyme assay. The deposit after centrifugation was resuspended in tris + saline buffer (pH 7·5) and turbidimetric readings made as previously described.

Cell walls suspended in de-ionized water were divided into two samples—one containing NaCl to a final concentration of 0·15 M and the other devoid of NaCl. Equal quantities of lysostaphin were added to each sample and turbidimetric readings recorded during the incubation period. The cell wall + lysostaphin mixture with no NaCl present was treated in the same manner after the 15 min. incubation as the viable cell + lysostaphin mixture.

RESULTS

Figure 1 indicated that in the absence of NaCl, minimum lysis of the staphylococci by lysostaphin occurred and the supernatant fluid of the suspension after centrifugation did not contain any lytic activity. A quantitative determination of lysostaphin as measured by lytic activity was obtained from the sediment after centrifugation and addition of NaCl, which was almost equivalent to the amount of enzyme initially added in the test.

Table 1. *Chemical analysis of 3684 µg. cell wall*

Wall components	µM	Ratio to glutamic acid	Calculated wt (µg) after correction for water of hydration
Aspartic acid	0·8	0·3	92·8
Threonine	0·2	0·07	20·4
Serine	2·0	0·7	176·2
Glutamic acid	2·8	—	364·3
Glycine	8·6	3	499·7
Alanine	5·6	2	451·4*
Lysine	2·6	1	335·9
Glucosamine	2·86	1	586·3†
Muramic acid	2·70	1	796·5†
			Total 3323·5

* One-half quantity is considered to be *c*-terminal amino acid and was not corrected for water of hydration.

† Adjusted on the basis of being *n*-acetyl derivative.

The method of purification of the cell walls resulted in a preparation that contained no detectable phosphorus or ribitol. *N*-terminal alanine, which one would expect to obtain from the labile *O*-alanyl ester linkage in teichoic acid (Armstrong, *et al.* 1958) was not present as dinitrophenylalanine. The amino acid analysis of the cell wall preparation (Table 1) showed the normal complement of amino acids and amino sugars reported in the staphylococci (Park, 1961) plus a small quantity of threonine and aspartic acid. The sum of the calculated weights of the constituents of the cell wall based upon the µM of material recovered after hydrolysis compared favourably with the initial dry weight of the cell walls used for the analysis.

The turbidimetric changes that took place when a suspension of cell walls was

mixed with lysostaphin is indicated in Fig. 2. In the absence of NaCl, a slight decrease in turbidity is exhibited during incubation. The saline suspension of the washed cell wall deposit obtained after centrifugation again exhibited a decrease in turbidity during incubation. The supernatant fluids obtained from the above centrifugation also contained active material as ascertained by its lytic action on washed *S. aureus* (sv). A total recovery of active material from the combined washings of the cell wall+lysostaphin suspension obtained before and after the addition of NaCl was not achieved.

Fig. 1. Lysis of viable staphylococci by lysostaphin. ●—● cocci with lysostaphin + NaCl; ○—○ cocci with lysostaphin without NaCl present; - - - cocci control with no NaCl present; *—* supernatant from cocci + lysostaphin mixture (without NaCl) added to viable cocci with NaCl present; □—□ deposit after centrifugation of cocci + lysostaphin mixture (without NaCl) resuspended with NaCl.

Electron micrographs (Pl. 1, figs. 1, 2) showed that the purified cell-wall preparation was attacked by the enzymic action of lysostaphin in that there was a conversion of the homogeneous cell-wall material to an irregular partially fragmented structure.

DISCUSSION

Since lysostaphin, by itself, does not sediment at 254,000g in a 4 hr period (Schindler & Schuhardt, 1965), the quantitative recovery of active lytic material from the washed deposit of a lysostaphin-viable *Staphylococcus aureus* mixture obtained after relatively low speed centrifugation indicates a complex being formed

between the enzyme and a component of the bacterial cell and that the addition of NaCl dissociated the complex. Weibull, Zacharias & Beckman (1959) found that under certain conditions lysozyme formed insoluble complexes with substances in the bacterial cell wall and the phenomenon which prevented lysis of the bacteria was interpreted in terms of a stable enzyme + substrate complex. Litwack (1960) in characterizing lysozyme and its substrate reported that complexes were formed with the teichoic acid of the cell wall as well as with the ribonucleic acids released from the lysed cells. He speculated that the stimulation of enzyme activity attributed

Fig. 2. Lysis of staphylococcal cell-wall mucopeptide. ●—● viable cocci with lyso-staphin + NaCl; □—□ mucopeptide with lysostaphin without NaCl; ○—○ supernatant of mucopeptide + lysostaphin mixture (without NaCl) with viable cocci and NaCl; *—* deposit after centrifugation of mucopeptide + lysostaphin mixture (without NaCl) resuspended with saline. Mucopeptide control showed no change in turbidity.

to NaCl during lysis of whole cocci could be due in large part to the liberation of nucleic acid-bound enzyme by the increased ionic strength of the medium.

The absence of detectable teichoic acid and the quantitative recovery of amino sugars and amino acids from the purified cell-wall preparation indicated that the material used in these experiments was essentially the mucopeptide moiety described by Park (1961) and by Rogers & Perkins (1962) and any complexes formed by the enzyme would be with this substrate and not with other anionic derivatives in the bacterial cell.

Admittedly, the molar ratio of alanine to glutamic acid in our preparation was

less than that reported present in the uridine nucleotide that accumulated with penicillin-treated *Staphylococcus aureus* (Park & Strominger, 1957). It is not known whether this discrepancy was due to strain differences or loss of components in the purification procedure. The presence of lytic material in both the supernatant fluid and the sediment of the mucopeptide + lysostaphin mixture appears to be at variance with the results obtained when whole cocci are combined with the enzyme in the absence of NaCl. This anomaly suggests that the action of the lytic protein on the cell-wall mucopeptide results in the release of complexes of varying molecular weight some of which are small enough to remain in suspension after centrifugation. With viable cocci, on the other hand, adhering cytoplasmic material and proteins confer sufficient weight to the complexes released into the medium to result in their being deposited on centrifugation.

Since the lytic protein is cationic under the experimental conditions used it is conceivable that the complexes formed are with anionic derivatives of the cell wall and the dissociation of the bonds by NaCl is similar to that reported by Mora, Young & Shear (1959) on the complexing of cationic macromolecules.

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EXPLANATION OF PLATE

Cell wall mucopeptide before (fig. 1) and after (fig. 2) addition of lysostaphin. Shadow cast with platinum + palladium (80 + 20).