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CELL-WALL PROTEINS OF *STAPHYLOCOCCUS AUREUS* : A KINETIC STUDY OF RELEASE BY LYSOSTAPHIN

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Résumé

LES PROTEINES DE LA PAROI DE *STAPHYLOCOCCUS AUREUS* : CINÉTIQUE DE LIBÉRATION PAR LA LYSOSTAPHINE. — La cinétique de lyse de la paroi de *Staphylococcus aureus* Cowan I a été suivie, à la fois par la mesure de la turbidité, par le dosage de certains composants, hexosamines, phosphore, protéines totales, protéine A, et par des observations de microscopie électronique.

Dans nos conditions expérimentales la lyse survient rapidement et, après cinq minutes, la structure caractéristique de la paroi n'est plus observée.

La libération des hexosamines, du phosphore et de la protéine A s'effectue de manière synchrone et se poursuit alors que la turbidité est devenue stable. La solubilisation de ces composants est complète en fin d'hydrolyse. Les protéines totales représentent environ 14 % du poids sec de la paroi. La protéine A est la seule protéine solubilisée au cours de l'hydrolyse.

Introduction.

The role of staphylococcal cell wall components in the human and animal infections (furonculosis, mastitis...) is not clearly known, though a number of studies underline their importance (Blobel *et al.*, 1976; Brückler *et al.*, 1975; Dossett *et al.*, 1969; Frost, 1975; Hasche *et al.*, 1975; Shayegani *et al.*, 1970; Targowski and Berman, 1975).

Among components, teichoic acids and peptidoglycan are the best known (Archibald, 1974; Schleifer and Kandler, 1972). On the other hand, there are few qualitative and quantitative data on cell-wall proteins (Yoshida *et al.*, 1963) except for protein A

and clumping factor (Jungerman, 1962; Sjöquist *et al.*, 1972; Switalski, 1976). For studying their role as well as that of other proteins which may be present, we must have these components in a pure state without using an extraction methods which may alter their biological activity. Thus, the enzymatic hydrolysis seems better than chemical extractions and hot extraction (Brückler *et al.*, 1974; Jensen, 1958; Jungerman, 1962; Switalski, 1976).

The lysostaphin which hydrolyses the peptidoglycan, the basic structure of the cell-wall (Browder *et al.*, 1965), releases protein A (Sjöquist *et al.*, 1972). The kinetic study of its action on the cell-wall under-

taken in this work should allow to specify both the conditions and interest of its use for isolation of the cell-wall proteins, in the view to a later biological study of these components.

Materials and methods.

Bacteria and growth conditions:

S. aureus Cowan I NCTC 8530 was grown on solid medium CCY 1 (Arvidson *et al.*, 1971) for 16 hr at 37°C.

Isolation of cell-walls:

The cells were harvested, resuspended in ammonium bicarbonate buffer 0.1 M, pH 7.8 (ABB) and heated at 80°C for 10 min, then washed three times (14,000 × g, 30 min., 4°C) with the same buffer. Disruption of cells was performed with glass beads (diameter 0.17-0.18 mm) in a Braun homogenizer (Zell-homogenisator MSK). The desintegrated cell suspension was treated overnight with 1% Triton X (Calbiochem) on a rotary shaker to release cytoplasmic membranes fragments (Sjöquist *et al.*, 1973). After centrifugation (14,000 × g, 30 min., 4°C), the pellet was washed three times with ABB by centrifugation. The yield of dried cell walls has been found to be approximatively 30% by weight of the whole cells.

Hydrolysis of cell-walls:

Cell-walls, 1.32 g (dry weight) were suspended in 120 ml of ABB. Lysostaphin, 0.7 mg (Schwarz-Mann) was added and the suspension was incubated at 37°C with a shaking water-bath.

Kinetic studies:

Two samples, 0.1 and 1 ml, of the incubation mixture were taken at various intervals: every 5 min. during the first 20 min., thereafter every 20 min. up to 500 min. The 0.1 ml sample was diluted to 1/100 with ABB (4°C) to measure turbidity at 600 nm. In order to stop hydrolysis the 1 ml sample was immediately diluted to 1/2 with ABB (4°C), centrifuged (10,300 × g, 10 min., 4°C);

the supernatant was filtered on 0.20 μ membrane (Sartorius). The filtrates were assayed for determination of cell wall components. The total mixture and the pellets at the beginning (time 0) and at the end (time 24 hrs) of hydrolysis were also assayed.

Total protein determinations:

The method of Lowry *et al.* (1951) was used for total mixture and pellets with a bovine serum albumin standard (British drug houses Ltd). Alternatively protein concentration of filtrates were estimated by measuring absorbance at 280 nm (aromatic amino acids) and at 232 nm (peptide bonds).

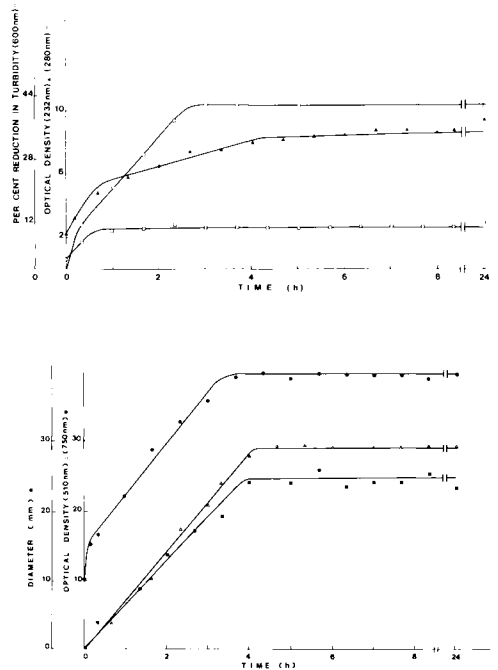


Figure 1: Cell wall hydrolysis kinetics by lysostaphin. Per cent reduction of turbidity at 600 nm (—○—○—○—). Proteins at 280 nm (—□—□—□—) and 232 nm (—▲—▲—▲—). Quantitative protein A determination by radial immunodiffusion (—●—●—●—); diameters of the circular precipitate were enlarged three times by projection before measurements Hexosamines at 510 nm (—△—△—△—). Phosphorus at 750 nm (—■—■—■—).

Table 1. Quantitative estimation of staphylococcal cell-wall components.

Total ⁽¹⁾	Components/dry weight of cell wall (% w/w)			
	Total proteins ^(2a)	Protein A	Hexosamines	Phosphorus
Before hydrolysis	13.8	—	24.5	1.82
After hydrolysis ⁽³⁾	14.5	—	22.3	2.3
Solubilized ⁽³⁾ (after 24 hrs of hydrolysis)	4.9	5.7 ⁽⁴⁾ - 4.6 ^(2b)	21.6	1.9

- (1) The total quantity of each component is evaluated both from the total suspension and from the sum of the values found for the pellet and the supernatant.
- (2) Estimation by the method of Lowry (2a: bovine serum albumin as reference ; 2b: pure Protein A as reference).
- (3) Values corrected taking into account the volumes removed during the experiment.
- (4) Estimation by radial immunodiffusion (pure Protein A as reference).

Protein A:

Single radial immunodiffusion (RID) according to Poutrel (1977) was performed for protein A determination: 15 µl of normal porcine serum pool was added to 13 ml of gel; volume of samples was 10 µl. Diameter of circular precipitate was measured after 48 h of incubation in a moist chamber at 15°C. Passive hemagglutination test was used for protein A detection (Flandrois *et al.*, 1975) on pellets. A possible proteolytic activity of lysostaphin (Oliver *et al.*, 1976) was tested by RID on pure protein A (Pharmacia).

Clumping-factor determination:

Passive hemagglutination test was used

for clumping-factor determination. Sheep red blood cells (SRBC, 2% v/v) were treated with glutaraldehyde (5% v/v Serva) according to Avrameas *et al.* (1969) to provide fibrinogen coated-cells (fibrinogen 5 mg/ml, Nutritional Biochemicals Corporation). Coated SRBC used in this test were diluted up to 0.8% (v/v) final concentration. The test, performed in microtiter plates was roughly quantitative.

Quantitative determination of hexosamines:

The amount of hexosamine was measured at 510 nm according to Elson-Morgan modified by Belcher *et al.* (1954) by reference to a standard curve prepared with glucosamine.

Quantitative determination of phosphorus:

The determination of phosphorus was performed at 750 nm according to Rouser *et al.*, (1970) after chromophore extraction with butanol.

Electron microscopy.

For negative staining, grids coated with collodion carbon films were floated face-downwards on cell-wall and hydrolysed cell wall preparations and then on a solution of 1% phosphotungstic acid (pH 7). The grids were dried by touching their edge with filter paper. Pellets of cell walls and hydrolysed cell walls were fixed as suspensions in 2% glutaraldehyde in 0.1 M cacodylate buffer (CB), pH 7.2, for 4 hrs at 20°C. After centrifugation the pellets were embedded in 2%

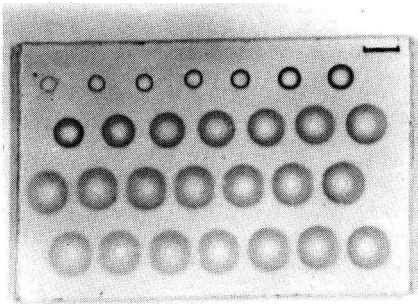


Figure 2: Kinetic of protein A release by radial immunodiffusion. Wells correspond to samples taken before hydrolysis, then according material and methods.

agar cut in small pieces and washed longer in CB at 20°C. The preparations were dehydrated in acetone and embedded in Epon (Finck, 1960). Thin sections were cut with glass knives on a Reichert OMU2 ultramicrotome and collected on carbon-collodion-coated copper grids.

The sections were stained with 4% uranyl acetate in methanol-water (1:1, v/v) and lead citrate (Venable and Coggeshall, 1965). Electron micrographs were taken in a Philips EM 300 electron microscope operating at 80 kV.

Results.

Cell-wall components release kinetics (figure 1).

Turbidity decreases according to the time of hydrolysis until the 3rd hour, then remains constant. The plateau corresponds to a reduction of 43% of the initial optical density.

Proteins are first rapidly released during 40 min., then absorbance at 280 nm does not vary any longer, whereas absorbance at 232 nm increases until the 4th hour proportionally to the time of hydrolysis.

The release of protein A (fig. 1 and 2), of hexosamines, of phosphorus occurs at the same speed and is proportional to the time of hydrolysis up to the maximum observed at the 4th hour.

The quantitative analysis of the cell wall and hydrolysate at 4 hrs shows a complete solubilisation of the protein A, hexosamines and phosphorus and partial solubilisation of the total proteins (33%, w/w) [Table].

The clumping-factor is found mainly in the hydrolysis pellets; it is present in traces only in the supernatant after 24 hours hydrolysis.

No proteolytic activity of the lysostaphin on the pure protein A (Pharmacia) has been observed in our experimental conditions.

Ultrastructure of the cell walls.

After disruption, staphylococcus, observed by the technique of the negative staining, appear as clear and flattened bacterial forms with released cytoplasmic material (Fig. 3a): which is mostly eliminated by Triton treatment (Fig. 3b). The presence of whole bac-

teria is rarely observed. In section, most of the cell walls are free from cytoplasm. Nevertheless, it is partially present associated with some rare cellular form (Fig. 3c). After 5 min. of hydrolysis, cell walls observed by the technique of negative staining do not have their characteristic form and only granulous masses are visible (Fig. 3d). After 24 hrs of hydrolysis, the pellet examined by the technique of thin section, does not show characteristic cell wall structures.

Discussion.

In our experimental conditions, the lytic activity of the lysostaphin is very fast; no characteristic cell wall structure can be seen after the fifth minute, as already reported (Schuhard *et al.*, 1969).

The synchronous solubilisation of hexosamines, protein A and phosphorus is a consequence of the cell wall destruction caused by peptidoglycan hydrolysis by the lysostaphin (Browder *et al.*, 1965). For these three components, the maximum solubilisation is reached after 4 hours of hydrolysis. On the other hand, turbidity doesn't change after the 3rd hour. This difference may be explained by the reduction of particle size in the suspension to a critical size which does not allow to measure a turbidity variation. The quantities of hexosamines, phosphorus that were report, are similar to the results of others (Huff *et al.*, 1964; Tipper and Strominger, 1966).

Our direct estimation of the total quantity of cell wall proteins by the Lowry technique (14% w/w) cannot be compared to that usually reported (30% w/w) (Yoshida *et al.*, 1963), because of the differences in the strain, conditions of culture and methodology. Yoshida *et al.* (1963) have employed an enzyme of undetermined lytic activity in their study of staphylococcal cell-wall. They characterized the material released only on the basis of sensitivity to proteolytic enzymes and spectrum absorbance and measured it by weight.

The quantity of protein A that we measured after hydrolysis of the cell wall, was similar to that reported (Lind, 1974; Sjöquist *et al.*, 1972). The protein A is no longer detected in the pellet after complete hydro-

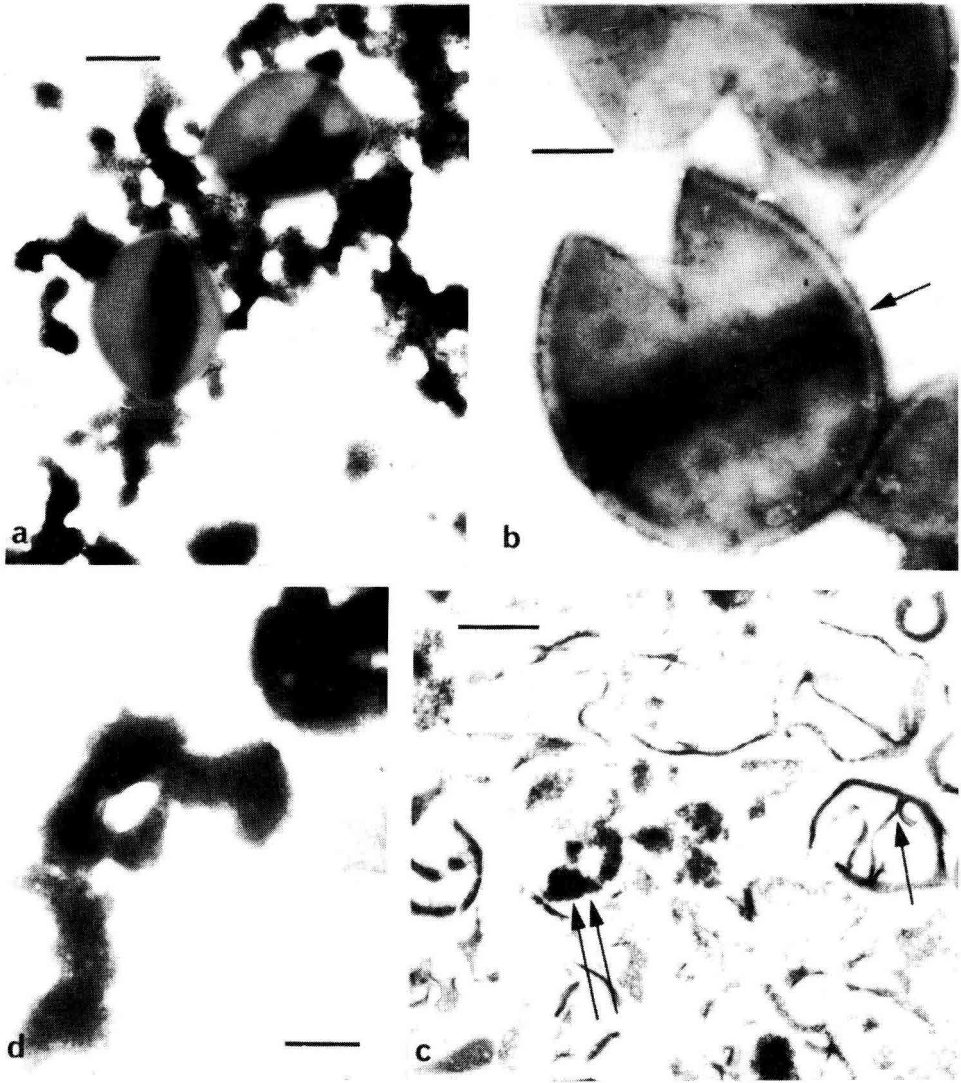


Figure 3a: Negatively stained preparation of disrupted cells of *Staphylococcus aureus*. Flattened envelopes were clearly seen with surrounding granulous cytoplasmic material. Bar: 500 nm.

Figure 3b: Negatively stained preparation of cells walls obtained after Triton treatment of disrupted cells. The cell wall with a septum (arrow) was free of cytoplasmic material. Granulous cytoplasmic material was rarely seen. Bar: 250 nm.

Figure 3c: Thin section of Triton cell walls. The cell wall was clearly seen with septum (arrow). Some cytoplasmic material was present (double arrows). Bar: 500 nm.

Figure 3d: Negatively stained preparation of 5 min hydrolysed cell walls. The characteristic flattened and spherical forms were not seen. Bar: 250 nm.

lysis. The Triton treatment of envelopes does not solubilize the protein A (Sjöquist *et al.*, 1972) furthermore, it is insensitive to a possible proteolytic activity of lysostaphin (Oliver *et al.*, 1976). These facts suggest to us that the total cell wall protein A has been solubilized and measured. The comparison of total solubilized protein quantities with protein A leads us to think that the protein A is probably the only solubilized protein. Such assumption is confirmed by the increase of absorbance at 232 nm associated with the constancy of absorbance at 280 nm. The absence of tryptophane residue and the reduced number of tyrosine residues in protein A may explain this dissociation (Sjöquist *et al.*, 1972).

The other proteins of the cell-wall, i.e. about 66%, are present in the hydrolysis pellet. The insolubilisation is probably due to their hydrophobic properties. It is not

possible to state that the clumping-factor is the only protein in this pellet. Some rare observations in electron microscopy do not allow us to ignore a possible contamination by proteins of cytoplasmic origin. Nevertheless, the fact that protein A is the only solubilized protein suggests low contamination.

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Summary.

The kinetics of cell-wall hydrolysis of *Staphylococcus aureus* (strain Cowan I) by lysostaphin was studied by turbidity, quantitative determination of hexosamines, phosphorus, total proteins, protein A and by electron microscopy.

In our experimental conditions, the lytic activity of lysostaphin was very fast and no characteristic cell-wall structures could be seen after five minutes.

The release of hexosamines, phosphorus and protein A was synchronous and could be recorded even though turbidity had become stable. At the end of the hydrolysis, the totality of these components was solubilized. Total proteins represented about 14% of the cell-wall dry weight. During hydrolysis protein A was the only protein solubilized.

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