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Elution of Loosely Bound Acid Phosphatase from *Staphylococcus aureus*[1]

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Strains of *Staphylococcus aureus* from the International-Blair and the Seto-Wilson series of phage propagating strains were examined for acid phosphatase activity. This enzyme was found to occur in varying amounts in three different fractions: free (6 to 60%), loosely bound (25 to 82%), and firmly bound (0 to 46%). Propagating strain 3A, because of its high activity, was chosen for further study. The rate of enzyme production paralleled cell growth in Trypticase Soy Broth, but followed a biphasic pattern in a semisynthetic casein acid-hydrolysate medium with glyceryl phosphate. Maximal elution of acid phosphatase in the loosely bound fraction, presumably from the surface of cells, occurred in the alkaline *p*H range. From log-phase cells, elution was maximally effected with buffered 1.0 M KCl (*p*H 7.5), but stationary-phase cells required twice the concentration of KCl.

Acid phosphatase activity has been demonstrated in many types of plant and animal cells; yet the enzyme has no known role in normal bacterial intermediary metabolism (3, 4). Phosphatases are known to catalyze hydrolytic and transfer reactions, and some evidence indicates that they are also involved in regulating cell permeability in some organisms (5). Use of phosphatases for concentrating inorganic phosphate in *Escherichia coli* (3), yeast cells (7), and algae (6) has been described. The widespread occurrence of acid phosphatase in nature, however, seems to indicate some function in those organisms in which it is found.

A demonstration of the localization of acid phosphatase in the bacterial cell may provide insight into the biological function of this enzyme (5). The localization of phosphatase in cells has been studied directly and indirectly. Mitchell (9) pointed out that perhaps more than 90% of the acid phosphatase of *Staphylococcus aureus* is found in the cytoplasmic membrane. Acid phosphatase has been eluted from *E. coli* (10) and yeast cells (18). Comprehension of the role of acid phosphatase at the molecular level in *S. aureus* requires first of all its elution from the cell and its solubilization in a stabilizing solvent. This study describes the widespread occurrence of acid phosphatase in *S. aureus* and the conditions necessary for one-step elution of the loosely bound fraction of the enzyme.

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MATERIALS AND METHODS

Cultures. A set of 25 propagating strains of the International-Blair Series (2) and of 5 propagating strains of the Seto-Wilson Series (15) of *S. aureus* cultivated in Brain Heart Infusion (Difco) was used to screen acid phosphatase activity. On the basis of its high activity, propagating strain 3A was selected for further studies in which Brain Heart Infusion was replaced by Trypticase Soy Broth (BBL) as reference medium.

Assay procedure. Acid phosphatase activity was assayed colorimetrically at *p*H 5.3 according to the method of Barnes and Morris (1). The unit of enzyme activity was expressed as micromoles of *p*-nitrophenol liberated per milliliter of sample at 37 C in 30 min.

Acid phosphatase screening (Brain Heart Infusion). Acid phosphatase screening was performed on 24-hr cultures (stationary) of *S. aureus* grown in Brain Heart Infusion at 37 C. Turbidity of each culture was adjusted to an optical density of 0.5, and enzyme activity was measured in the presence of 0.01% thimerosal. Cells grown in the manner previously described were harvested and centrifuged. Depending upon the ease of dissociation from the cells, three forms of acid phosphatase were identified. Acid phosphatase detached from the cells and dissolved in the cell-free supernatant fluid was designated "free" acid phosphatase. The sedimented cells were washed six times by resuspension in 0.15 M NaCl solution and subsequent recentrifugation. The total enzyme in the combined washings was termed "loosely bound." Enzyme activity in the sixth washing was not measurable. The designation "firmly bound" was reserved for the residual activity still associated with the cells after the salt washing series.

Acid phosphatase production in undefined (Trypticase Soy Broth) and semidefined media. The rate of

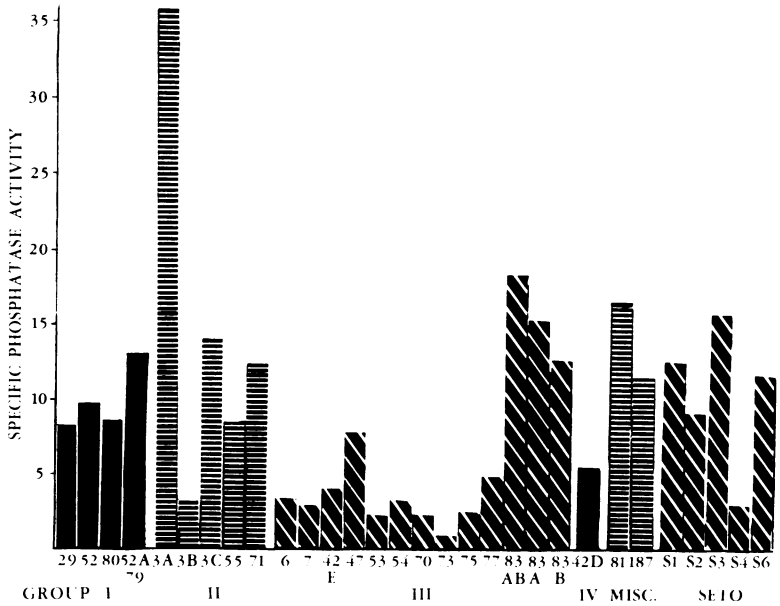

FIG. 1. *Specific acid phosphatase activity of whole cultures of Staphylococcus aureus (samples adjusted to optical density of 0.5) cultivated in Brain Heart Infusion for 24 hr under stationary conditions. Cultures were selected from the International-Blair and Seto-Wilson series of phage propagating strains.*

whole culture and free acid phosphatase production and cell density (read at 625 mμ) were determined during shake cultivation at 37 C in a 1-liter quantity of *S. aureus* PS 3A in Trypticase Soy Broth. These same parameters were measured also in cultures grown in the casein acid-hydrolysate medium of Stutzenberger, San Clemente, and Vadehra (16) with the following modifications: 0.05% glyceryl phosphate was substituted for the inorganic phosphate salts, and 0.05 M tris(hydroxymethyl)aminomethane (Tris)chloride (*p*H 7.2) was used to buffer the medium. Additionally, residual glucose was determined by the method of Noelting and Bernfeld (12).

Acid phosphatase elutions. To determine the optimal *p*H range of elution, three samples (20 ml each) of actively dividing cells of *S. aureus* PS 3A grown in Trypticase Soy Broth were centrifuged and resuspended in equal volumes of 0.1 M buffer solutions at *p*H 5.3 (acetate), 7.0, and 8.5 (Tris chloride). These suspensions at each *p*H value were incubated at 37 and at 25 C for 2 hr, and the cells were harvested by centrifugation. Each sample was washed with 0.15 M NaCl, the cells were resuspended in the same salt solution, and all fractions were assayed for enzymatic activity. Ionic elution of acid phosphatase from *S. aureus* PS 3A was carried out when washed log-phase cells grown in Trypticase Soy Broth and stationary-phase cells grown in the casein acid-hydrolysate medium were suspended in solutions varying from 0.1 to 2.0 M KCl at *p*H 7.5 for 60 min at 25 C. The cells were concentrated by centrifugation and resuspended in water after further washings. Cell-free culture medium was assayed for free enzyme. Each salt and wash solution was assayed after dialysis against 0.1 M Tris chloride buffer (*p*H 7.5) at 4 C for enzyme activity of the loosely bound fraction. Likewise, cells resuspended in water were assayed for enzyme activity of the firmly bound fraction. Dry weight and viable count determinations were made on the whole culture and on the final cell suspensions in water to correct for any cell loss during the eluting process.

RESULTS

Acid phosphatase screening. All phage-propagating strains of *S. aureus* selected from the International-Blair and Seto-Wilson series showed acid phosphatase production after 24 hr of growth. Subsequently, when all samples were adjusted to an optical density value of 0.5, PS 3A surpassed all others in phosphatase activity (Fig. 1). High enzymatic activity was not confined to any particular phage-propagating group. Relative amounts of acid phosphatase in the free, loosely bound, and firmly bound fractions of the phage-propagating strains are shown in Fig. 2. Free phosphatase activity in spent culture medium ranged from 6% in PS 52A/79 to 60% of the total activity in propagating strains 52, 3C, 6, and 73. Enzymatic activity in the loosely bound fraction was relatively higher and ranged from 25% in PS 7 to 82% in PS 70. Other propagating strains demonstrating high activity in this fraction included 3A, 55, 77, and 42D of the International-Blair series and S1 of the bovine-adapted series.

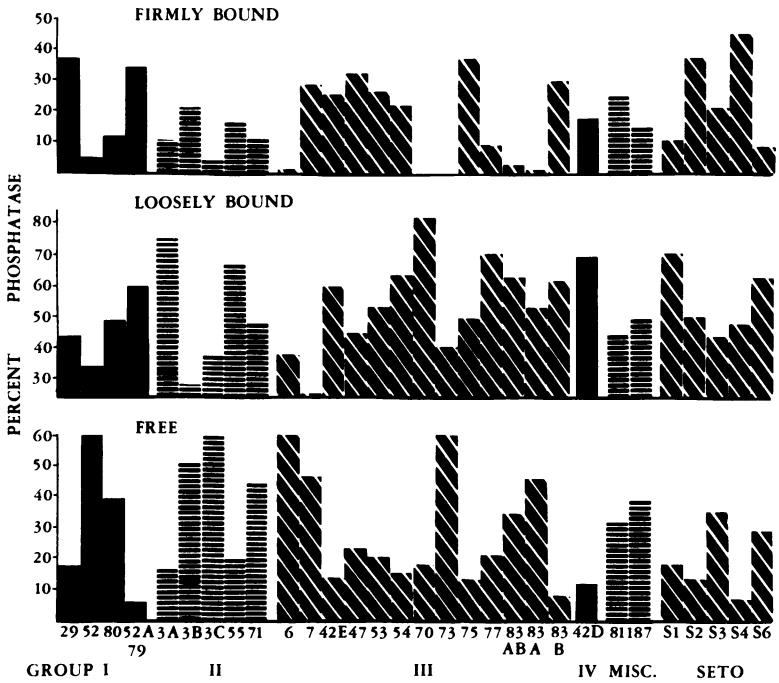

FIG. 2. *Relative amounts of acid phosphatase in terms of free, loosely bound, and firmly bound fractions taken from BHI cultures of the International-Blair and Seto-Wilson series of phage propagating strains of Staphylococcus aureus.*

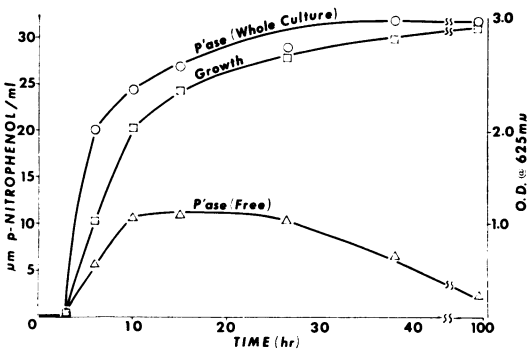

FIG. 3. *Rate of acid phosphatase (P'ase) production (whole culture and cell-free supernatant fluid) and cell growth measured in shake cultures (Trypticase Soy Broth) of Staphylococcus aureus PS 3A at 37 C.*

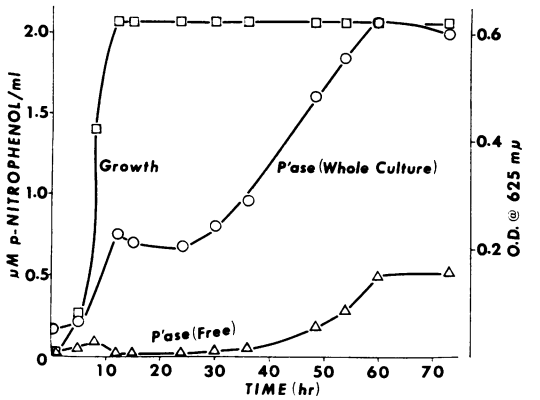

FIG. 4. *Rate of acid phosphatase (P'ase) production (whole culture and cell-free supernatant fluid) and cell growth measured in shake cultures (casein acid-hydrolysate medium with glyceryl phosphate) of Staphylococcus aureus PS 3A at 37 C.*

In general, minimal acid phosphatase activity was associated with the firmly bound fraction of *S. aureus*, with no apparent activity in both PS 70 and PS 73 to 46% of the total activity in PS S4.

Acid phosphatase production in undefined and semidefined media. The rate of whole culture acid phosphatase production in Trypticase Soy Broth paralleled cell density of a shake culture of PS 3A (Fig. 3). However, free enzyme activity was maximally obtained after 10 hr of growth and started to decline 16 hr later. In the casein acid-hydrolysate medium containing glyceryl phosphate, the rate of whole culture acid phosphatase production also increased with cell number (Fig. 4), but the total amount of phosphatase produced was about one-fifteenth of that elaborated in

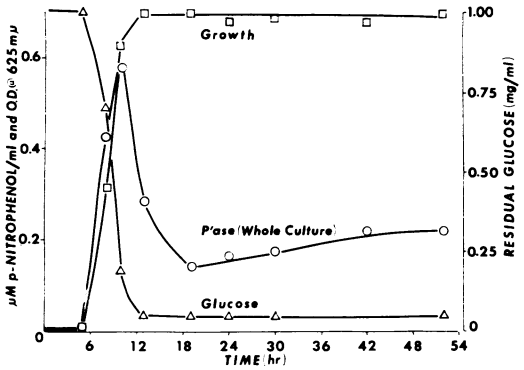

FIG. 5. *Rate of whole culture acid phosphatase (P'-ase) production, glucose utilization, and cell growth measured in shake cultures (casein acid-hydrolysate medium without glyceryl phosphate) of Staphylococcus aureus PS 3A at 37 C.*

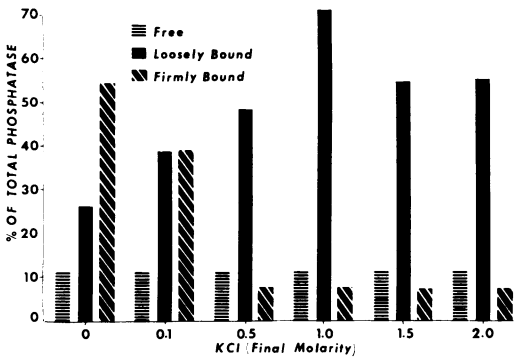

FIG. 6. *Relative amounts of free, loosely bound, and firmly bound acid phosphatase in shake cultures of Staphylococcus aureus PS 3A, cultivated in Trypticase Soy Broth at 37 C. Enzyme elution was effected with varied concentrations of KCl at pH 7.5.*

Trypticase Soy Broth (Fig. 3). However, 18 hr later enzyme production again increased after a period of lag. Free enzyme activity exhibited essentially the same pattern. In the absence of glyceryl phosphate, no substantial increase was noted in acid phosphatase production during the stationary phase of the growth curve (Fig. 5). Depletion of glucose coincided with the termination of exponential cell division.

Acid phosphatase elutions. Maximal enzyme occurred in the alkaline *p*H range (Table 1). Acid phosphatase was equally eluted at 25 or 37 C at neutral or slightly alkaline conditions. Since the enzyme appeared more labile when left in a highly basic menstruum for long periods of time, *p*H 7.5 was selected for routine application. Of the acid phosphatase found associated with actively dividing cells of PS 3A grown in Trypticase Soy Broth, 70% was loosely bound to the cells (Fig. 6). The same graph shows that the enzyme was maximally eluted from the cells with

TABLE 1. *The pH dependence of acid phosphatase elution from cells cultivated in shake cultures (Trypticase Soy Broth) of Staphylococcus aureus PS 3A, with 0.1M buffer solutions at 25 and 37 C*

Fraction	Activity[a]					
	Elution *p*H at 37 C			Elution *p*H at 25 C		
	5.3	7.0	8.5	5.3	7.0	8.5
Eluted	2.82	1.90	8.11	0.10	1.50	9.80
Retained	25.60	32.00	23.00	25.50	31.60	27.60

[a] Expressed as micromoles of *p*-nitrophenol per milliliter per 30 min at 37 C.

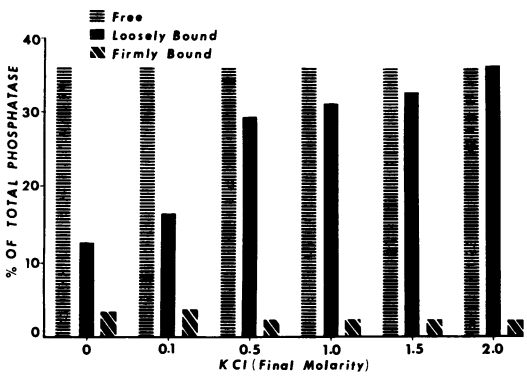

FIG. 7. *Relative amounts of free, loosely bound, and firmly bound acid phosphatase in shake cultures of Staphylococcus aureus PS 3A, cultivated at 37 C in a casein acid-hydrolysate medium supplemented with glyceryl phosphate. Enzyme elution was effected with varied concentrations of KCl at pH 7.5.*

1.0 M KCl at *p*H 7.5, and that the amount eluted increased as a function of ionic strength up to 1.0 M. However, the elution pattern of acid phosphatase was different in the case of stationary-phase cells of PS 3A grown in the casein acid-hydrolysate medium (Fig. 7). In this case, relatively higher enzyme activity was found in the free fraction, and maximal elution of the loosely bound fraction occurred at a final concentration of 2.0 M KCl. When whole culture activity was used as reference, the acid phosphatase recovered was greater during the log phase than the stationary phase.

DISCUSSION

Experimental evidence indicates no restriction of high phosphatase activity to any particular

group of phage-propagating strains of coagulase-positive staphylococci. Relatively little alkaline phosphatase activity was observed in the strains of *S. aureus* used in our studies.

When *S. aureus* PS 3A is grown in complex media, the rate of whole culture acid phosphatase production is a function of cell number. Barnes and Morris (1) made the same observation on *S. aureus* cultivated on nutrient agar. Production of acid and alkaline phosphatases by *E. coli* (17) seems to be more complex; acid phosphatase is always present, but alkaline phosphatase is formed only when the concentration of inorganic phosphorus is limiting in the medium. Hofsten (14) later describes a repressive effect of carbohydrates on acid phosphatase production in *E. coli*. In our study (Fig. 4), when *S. aureus* was grown in a semisynthetic medium in the presence of glyceryl phosphate, there was an unexpected increase in enzyme activity during the stationary phase of the growth curve. This increase in activity cannot be due to increased cell number since cessation of cell division coincides with glucose depletion from the medium. Increased phosphatase activity is probably the result of enzyme induction, since a similar increase in activity is not observed in the absence of glyceryl phosphate (Fig. 5).

Elution of acid phosphatase from *S. aureus* is a function of *p*H and ionic strength. Salt concentrations of 1.0 to 2.0 M at alkaline *p*H values are optimal for elution of the enzyme. Our system for enzyme elution necessitates a higher salt concentration than that of 0.5 M KCl reported for acid phosphatase elution from *Saccharomyces mellis* (18). The enzyme of yeast cells could not be eluted at higher salt concentrations unless a thiol compound were added to the eluting menstruum. The same authors later report that great variation for conditions of acid phosphatase elution exists among related species of yeast cells (19).

Elution of acid phosphatase with a salt solution indicates the enzyme is associated at least in part with the cells through electrostatic interactions. The fraction eluted by salt solution is thought to be loosely bound to the cell surface and probably gives rise to the free fraction found in the growth medium. Rogers (14) suggests that extracellular enzymes of *S. aureus* are extruded into the growth medium as a capsular material that later dissolves.

In the case of yeast (18), a firmly bound acid phosphatase is removed only by wall digestion with a snail-gut extract. This technique was not applied to our organism, nor was the nature of the firmly bound acid phosphatase investigated in the present study. However, we did find that this fraction accounts for 0 to 40% of the total enzyme activity.

Solubilization of acid phosphatase in a protein-free ionic solution provides us with a major step in enzyme purification and with some insight into localization of the enzyme within the cell. The loosely bound fraction is probably located on the surface of the cell, but more direct evidence is needed to localize the firmly bound fraction. Neu and Heppel (11) suggested the existence of a family of degradative enzymes on the cell surface of *E. coli*.

The role of acid phosphatase in microorganisms is still a matter of conjecture. The enzyme in *S. aureus* may play some important biological and ecological role, since some strains produce significant amounts of the enzyme. Acid phosphatase may well be a part of the "translocase" mechanism involved in phosphate transfer across the cell membrance as proposed by Mitchell (8). A permeability role for acid phosphatase has been suggested for *E. coli* (5). On the other hand, Pauwels (13) reports that phosphatases in yeast cells have only a minor role in phosphate absorption, and that these enzymes do not limit the process of absorption. Thus, it seems that the phosphatases have diverse roles in different microorganisms just as enzyme localization and requirements for enzyme elution seem to differ. A more complete knowledge of the role of acid phosphatase in *S. aureus* would require its solubilization, purification, and the study of its molecular properties.

ACKNOWLEDGMENT

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