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Staphylococcal Acid Phosphatase: Extensive Purification and Characterization of the Loosely Bound Enzyme[1]

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Acid phosphatase of *Staphylococcus aureus* PS55 was eluted from the surface of these cells with 1.0 M KCl at *p*H 8.5 by gentle agitation at 25 C and was purified 44-fold (51% recovery) by two cycles of dialysis and gel filtration. The eluted enzyme which had a 280/260 (nm) absorbancy ratio of 0.71 required at least 0.5 M salt solution for solubilization; however, most of the purified product which had a 280/260 (nm) absorbancy ratio of 1.72 was soluble in dilute buffer solution [0.01 M tris(hydroxymethyl)aminomethane chloride, *p*H 8.5]. Purified acid phosphatase appeared homogeneous according to the criteria of gel filtration, starch-block electrophoresis, and analytical ultracentrifugation. In a starch block, migration was toward the cathode at *p*H 8.0. Maximal activity occurred at *p*H 5.2 to 5.3 and salt concentration had little effect on phosphatase activity up to 1.0 M KCl or NaCl. Progressive loss of enzymatic acitivity occurred at higher salt concentrations. Molecular weight of purified acid phosphatase was estimated to be 58,000.

Phosphatase has sometimes been implicated as a virulence factor for *Staphylococcus aureus* (9, 10, 13). The organism produces both an acid and alkaline phosphatase, the latter being repressed by inorganic phosphate in the growth medium (19). However, acid phosphatase is not repressed by inorganic phosphate, but rather is induced by glycerophosphate in a semisynthetic medium (15).

The acid phosphatase of *Escherichia coli* is a constitutive enzyme, but alkaline phosphatase is formed only when inorganic phosphorus in the medium is limiting (21). More recently, Dvorak et al. (8) demonstrated that acid phosphatase of *E. coli* is subject to catabolite repression, and enzymatic activity is significantly reduced by glucose, glucose 6-phosphate, glycerol, and glycerophosphate. The same investigators isolated three separate acid phosphatases from *E. coli* and purified one phosphatase which was highly specific for certain hexose and pentose phosphate esters.

The widespread occurrence of acid phosphatase in *S. aureus* and the conditions necessary for one-step elution of this loosely bound enzyme have already been reported (15). Since the enzyme is eluted with a salt solution, it is thought to be loosely bound to the cell surface, at least in part, by electrostatic interactions. Neu and Heppel (17) suggested the existence of a family of degradative enzymes on the surface of *E. coli*.

There is no information on the purification of staphylococcal acid phosphatase, and only limited characterizations of the enzyme have been made (2). This study was undertaken to purify and characterize acid phosphatase to gain knowledge of the possible function of this enzyme in the staphylococci.

MATERIALS AND METHODS

Culture. Phage-propagating strain 55 of *S. aureus* from the International-Blair series (4) was used throughout this study. Stock cultures were maintained on Trypticase Soy Agar (BBL) slants at 4 C and were transferred every 6 weeks.

Acid phosphatase production. A 500-ml log-phase culture of PS55 grown in Trypticase Soy Broth (BBL) was used to seed 16 liters of the same medium. Cultures were then grown at 37 C on a rotary shaker for 16 to 20 hr. Samples of the whole culture were taken for acid phosphatase and dry-weight determinations. Cells were then harvested at 25 C by continuous-flow centrifugation.

Purification of acid phosphatase. The cells were washed with 0.1 M KCl in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (*p*H 8.5), cen-

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trifuged, and resuspended in 400 ml of 1.0 M KCl in 0.5 M Tris-hydrochloride (*p*H 8.5). This latter treatment effected elution of loosely bound enzyme from the cells as previously reported (15). Routinely, acid phosphatase was eluted by gentle agitation on a reciprocal shaker at 25 C for 60 min. The eluted material was then dialyzed against 10 volumes of 0.01 M Tris-hydrochloride for 12 hr at 4 C. The precipitate formed during dialysis was sedimented in a refrigerated (4 C) centrifuge, redissolved in 1.0 M KCl—0.5 M Tris-hydrochloride solution, and was redialyzed under the conditions already described. The precipitate formed after the second dialysis was again dissolved in 1.0 M KCl—0.5 M Tris-hydrochloride solution, layered on a column (2.5 by 38 cm) of Sephadex G-100, and eluted from the column with 1.0 M KCl in 0.05 M Tris-hydrochloride buffer, *p*H 8.5. Fractions (4.3 ml) were collected at 5 C and scanned at 280 and 260 nm to locate eluted protein. Those fractions containing protein were assayed for acid phosphatase activity. Certain tubes containing maximal enzymatic activity were combined and dialyzed against 0.01 M Tris-hydrochloride (*p*H 8.5) for 12 hr at 4 C. The precipitated material was redissolved in 1.0 M KCl—0.5 M Tris-hydrochloride solution and was rechromatographed as previously described. The collected fractions were again assayed for protein and enzymatic activities, and certain tubes with maximal enzymatic activity were combined. All characterizations were performed on this purified product.

Qualitative and quantitative methods. Acid phosphatase activity was measured routinely by a method modified from Barnes and Morris (2). The reaction mixture, contained in a total volume of 3.0 ml, consisted of 1.4 ml of 0.1 M acetate buffer (*p*H 5.2), 1.0 ml of sample, and 0.6 ml of 0.4% (w/v) *p*-nitrophenol phosphate, disodium salt (Mann Research Laboratories, Inc., New York, N.Y.) as substrate. After 30 min of incubation at 37 C, the reaction was stopped with 1.0 ml of 1 M NaOH; the resulting color was measured spectrophotometrically at 400 nm. When adequate controls were used, it was not necessary to eliminate residual color (due to the presence of extraneous material, not *p*-nitrophenol) with HCl. The unit of enzyme activity was expressed as micromoles of *p*-nitrophenol liberated per min at 37 C.

Protein determinations were made according to the method of Lowry et al. (14), with crystallized, bovine fraction V albumin used as protein standard. When high salt concentrations prohibited use of this colorimetric technique, protein measurements were made spectrophotometrically according to the method of Warburg and Christian (22).

Numerous techniques were employed to detect the presence or absence of associated staphylococcal products during the purification of acid phosphatase. Coagulase was measured by the titer method of Tager and Hales (20) at 37 C. Deoxyribonuclease activity was analyzed by placing 0.2 ml of each purified fraction inside separate wells cut into DNase Test Agar (Difco). Treated plates of agar were incubated at 37 C for 12 hr and then flooded with 1 N HCl. Hemolysins were detected by streaking 0.2 ml of each purified fraction onto sheep blood- and human blood-agar plates. The plates were incubated for 8 hr at 37 C and observed for hemolysin activity. Lipase activity was assayed according to the method of Nachlas and Seligman (16) using *p*-nitrophenyl palmitate (Sigma Chemical Co., St. Louis, Mo.) as substrate. The reaction occurred in Veronal buffer (*p*H 7.4) at 40 C. After a 1-hr incubation period, samples were assayed for chromogenic product. Fibrinolysin activity was analyzed after 12 hr at 37 C on fibrin plates which were made by adding citrated bovine fibrinogen (Sigma) to warm agar. Carbohydrate was detected by the Molish test (6) and nucleic acid by the method of Warburg and Christian (22).

Homogeneity of purified acid phosphatase. Electrophoretic homogeneity was determined by starch-block electrophoresis. Starch preparation and electrophoresis were performed according to the methods of Campbell et al. (5, p. 57–62). Approximately 0.5 mg of protein in 0.2 ml of solution was applied to the starch block. When electrophoresis was completed (after 12 hr at 4 C), the starch block was cut into 1-cm-wide segments, from which the enzyme was eluted with 2 ml of cold 0.15 M NaCl. Each fraction was analyzed for protein and acid phosphatase.

Analytical ultracentrifugation was carried out in a Spinco model E analytical ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) by the sedimentation velocity method at 4 C. The purified enzyme (0.4 ml), containing 3.5 mg of protein per ml, was placed in a single sector synthetic boundary cell along with 0.1 ml of solvent (0.6 M KCl in 0.1 M Tris-hydrochloride, *p*H 8.5). The run was made at 56,100 rev/min for 80 min using an An-D analytical rotor. The bar angle was 65°, and pictures were taken at certain time intervals. Calculations of $S_{20,w}$ were done according to the method of Schackman (18).

Void volume of the chromatographic column was determined with Blue Dextran 2000 at 5 C. The pore radius for the gel was determined by using cytochrome *c*. Calculation of the diffusion coefficient for acid phosphatase at standard conditions was done according to the method of Ackers (1).

Effect of pH on the activity of acid phosphatase. Enzymatic activity of purified product was analyzed between *p*H 4 and 10. The following buffers were employed in the assay system: 0.1 M acetate (*p*H 4.0 to 5.6); 0.1 M sodium arsenate-HCl (*p*H 6.5); 0.1 M Tris-hydrochloride (*p*H 7.3 to 8.5); and 0.1 M glycine-NaOH (*p*H 9.4 to 10.0). All samples were incubated at 37 C for 30 min.

Effect of salt concentration on the activity of acid phosphatase. The effect of both KCl and NaCl on enzymatic activity was measured in a series of assay tubes in which the final salt concentration ranged from 0.01 to 2.0 M. Buffer concentration in all cases was 0.001 M acetate. Two separate controls were employed: one at each salt concentration in which the reaction was stopped at zero-time, and the other which contained no added salt.

RESULTS

Purification of acid phosphatase. In a previous report (15), we defined "loosely bound" acid

phosphatase as that fraction which is eluted from the cells by washing them in a salt solution such as KCl. The "firmly bound" enzymatic activity resisted ionic elution with KCl. The whole culture usually contained 0.50 to 0.67 units of enzymatic activity per ml, and the cells alone normally accounted for 50 to 60% of this activity. In routine purification experiments, the eluted fraction (loosely bound) of acid phosphatase had a specific activity 5 to 10 times greater than the cells alone. The 280/260 (nm) absorbancy ratio of this eluted fraction was 0.71.

During dialysis of eluted enzyme against dilute buffer (*p*H 8.5), acid phosphatase was precipitated. At salt concentrations less than 0.5 M, solubility of the enzyme rapidly decreased (Fig. 1), and at 0.2 M, more than 90% of the enzymatic activity was precipitated. After the enzyme was redissolved in 1.0 M KCl (*p*H 8.5), this fraction displayed a specific activity of 43.4 which represented a 24-fold purification of enzyme. The 280/260 (nm) absorbancy ratio was 0.87, and there was an unexpected increase in per cent recovery over the previous purification step.

Figure 2 illustrates a typical elution pattern of acid phosphatase and protein when the redissolved precipitate (obtained after the second dialysis step) was passed through a column of Sephadex G-100. One peak of acid phosphatase activity and two protein peaks were observed. The partition coefficient (K_{av}) was 0.2 for the major protein peak and 0.6 for the minor protein peak. The major peak exhibited acid phosphatase activity, and the minor peak had deoxyribonuclease activity but no acid phosphatase activity.

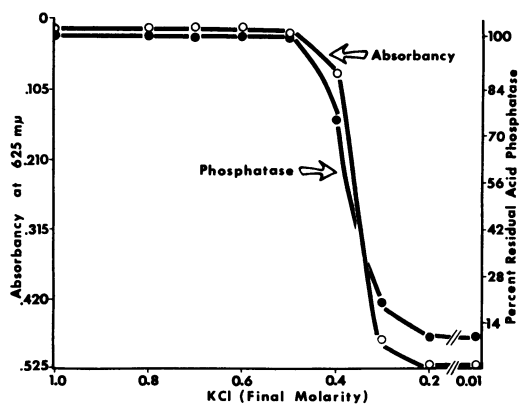

FIG. 1. *Solubility curve of acid phosphatase (precipitate formed after second dialysis step) prior to gel filtration. Decreasing solubility of the enzyme was determined by observing (at 625 nm) the precipitate formed, and residual enzymatic activity was determined by assaying the supernatant fluid.*

Figure 3 represents the elution pattern after recycling through Sephadex G-100 the fractions which demonstrated maximal phosphatase activity. The K_{av} for the common protein and acid phosphatase peak was 0.2. The pore radius for the gel was 18.1 nm and Stoke's radius for acid phosphatase was 5.53 nm. Diffusion coefficient (at standard conditions) was calculated to be 5.43×10^{-7} cm^2 sec^{-1}.

Efficiency of the purification procedure is shown in Table 1. As seen here, the purified product obtained after the second cycle of gel filtration accounted for 51% of the loosely bound acid phosphatase. A specific activity of 78.4 represented approximately a 44-fold purification. The 280/260 (nm) absorbancy ratio of purified acid phosphatase was 1.72.

Presence or absence of other staphylococcal products. Table 2 illustrates the presence or absence of some staphylococcal products during different stages of purification. Acid phosphatase, coagulase, lipase, deoxyribonuclease, and nucleic acid were simultaneously removed from the cells

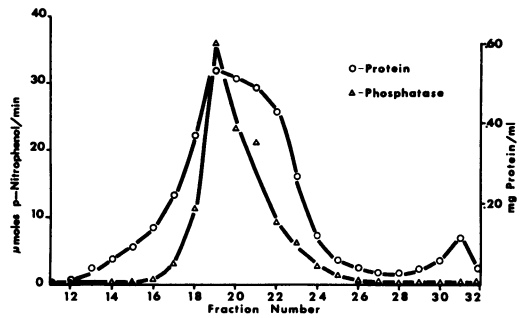

FIG. 2. *Gel filtration (Sephadex G-100) of the fraction precipitated after the second dialysis step using a column (2.5 by 38 cm) at 5 C. Enzyme was eluted with 1.0 M KCl in 0.05 M Tris-hydrochloride buffer, pH 8.5.*

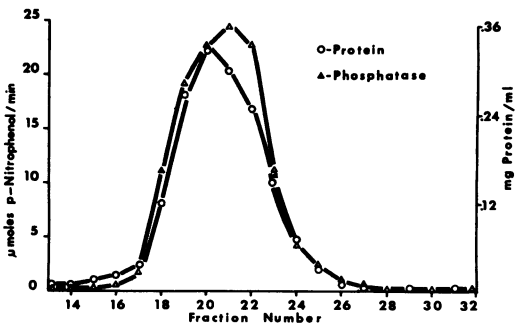

FIG. 3. *Gel filtration (Sephadex G-100) of the fraction dialyzed after the first gel filtration using a column (2.5 by 38 cm) at 5 C. Enzyme was eluted with 1.0 M KCl in 0.05 M Tris-hydrochloride buffer, pH 8.5.*

TABLE 1. *Enzyme purification summary for staphylococcal acid phosphatase*

Purification fraction	Specific activity[a]	Per cent recovery	280/260	Purification factor
Eluted phosphatase	1.8		0.71	
First dialysis	43.4	188	0.87	24.1
Second dialysis	45.0	194	0.90	25.0
First gel filtration	52.6	65	1.43	29.2
Second gel filtration	78.4	51	1.72	43.5

[a] Expressed as micromoles of *p*-nitrophenol per minute per milligram of protein.

TABLE 2. *Presence or absence of some staphylococcal products during purification of acid phosphatase*

Staphylococcal product	Purification fraction				
	Eluted	First dialysis	Second dialysis	First filtration	Second filtration
Acid phosphatase	+	+	+	+	+
Coagulase	+	−	−	−	−
Lipase	+	±	−	−	−
Deoxyribonuclease	+	+	+	−	−
Hemolysin	−	−	−	−	−
Fibrinolysin	−	−	−	−	−
Carbohydrate	−	−	−	−	−
Nucleic acid	+	+	+	+	−

with 1.0 M KCl. Carbohydrate was not removed from the cells which were initially devoid of hemolysin and fibrinolysin activities. During dialysis against dilute buffer, phosphatase, deoxyribonuclease, and nucleic acid were precipitated; coagulase and lipase activities remained soluble. The first cycle of gel filtration eliminated nuclease, and the second cycle increased the 280/260 (nm) absorbancy ratio to 1.72 by removing contaminating nucleic acid.

Electrophoretic homogeneity. Electrophoretic analysis on a starch block indicated that the purified preparation was homogeneous with respect to charge (Fig. 4). The basic nature of acid phosphatase was apparent from its migration (2 cm from the origin) toward the cathode at *p*H 8.0.

Sedimentation constant and molecular weight. Figure 5 illustrates the sedimentation pattern of acid phosphatase 40 min after the rotor reached maximal speed. Sedimentation was from left to right. Since only one symmetrical peak was

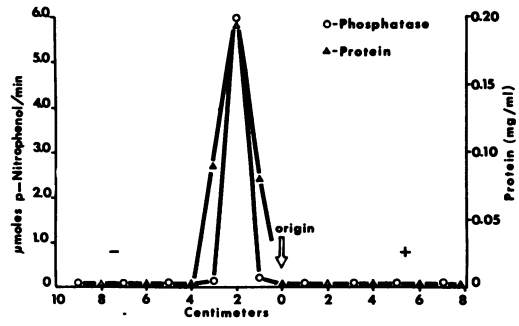

FIG. 4. *Migration of purified acid phosphatase in starch-block electrophoresis at pH 8.0 and 4 C after 12 hr. Protein and enzymatic activities were determined in the material eluted from fractions consisting of 1-cm wide segments which were cut from the starch block perpendicular to the direction of migration.*

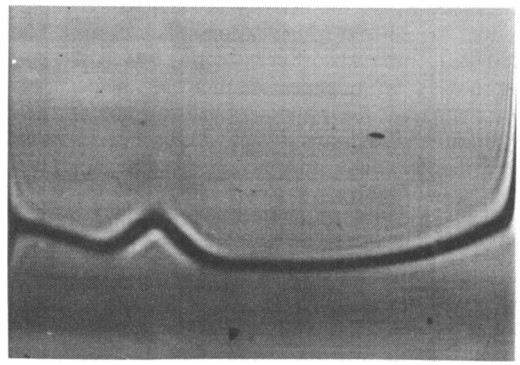

FIG. 5. *Sedimentation pattern of partially purified acid phosphatase. The enzyme was dissolved in 0.6 M KCl—0.1 M Tris, pH 8.5. The photograph was taken 40 min after the rotor had reached maximal speed of 56,100 rev/min. The bar angle was 65°; protein concentration was 2.8 mg/ml.*

obtained, the preparation probably contained a homogeneous species of acid phosphatase. The $S_{20,w}$ in this case was 3.68. Molecular weight of the purified enzyme, estimated according to the method of Ackers (1), was 58,000.

Effect of pH on the activity of acid phosphatase. The range of optimal *p*H for the activity of acid phosphatase was 5.2 to 5.3 (Fig. 6). A decrease of one *p*H unit from the optimal value resulted in about 50% reduction in enzymatic activity; an increase of one unit caused a 30% reduction. The enzyme exhibited little or no activity in the alkaline *p*H range.

Effect of salt concentration on the activity of acid phosphatase. The optimal salt concentration for acid phosphatase activity was in the range 0.2 to 0.5 M (Fig. 7). With 1.0 M KCl or NaCl, 80% of the optimal activity was still present;

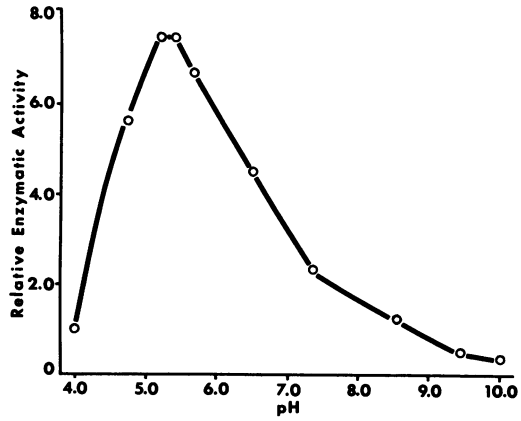

FIG. 6. *Effect of pH on the activity of acid phosphatase using p-nitrophenyl phosphate as substrate.*

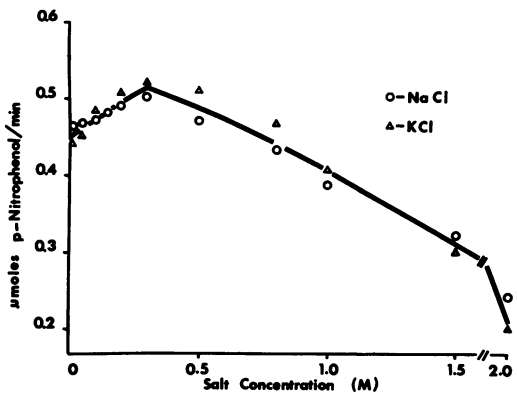

FIG. 7. *Effect of different salt concentrations on the activity of acid phosphatase. Buffer (pH 5.2) concentration in all cases was 0.001 M acetate.*

with 2.0 M, less than half the optimal enzymatic activity was apparent.

DISCUSSION

We were able to purify staphylococcal acid phosphatase 44-fold by employing the mild procedures of elution, dialysis, and gel filtration. Solubilization of the enzyme in a protein-free ionic solution (15) provided us with a major step in enzyme purification. Neu and Heppel (17) recognized that a good first step in purification of an enzyme is its selective removal from the cells. They eluted certain enzymes from *E. coli* by an "osmotic shock" procedure. Our elution procedure yielded a 5- to 10-fold purification of acid phosphatase when compared to untreated cells. With *E. coli*, the osmotic shock fluid containing an acid phosphatase gave a 30-fold purification when compared with sonic extracts (8).

A 280/260 (nm) absorbancy ratio of 1.72 for our final product indicated the purified material was essentially free from contaminating nucleic acid, and 51% of the loosely bound enzyme was recovered. The precipitated enzyme obtained after the first dialysis displayed a higher recovery (188%) than did the eluted material. We attributed this phenomenon to one or both of the following possibilities: (i) removal of some small molecular weight inhibitor(s) during dialysis; (ii) disaggregation of acid phosphatase from other macromolecules that remain soluble during dialysis against dilute buffer while phosphatase is precipitated. Such aggregates could function by masking the active site(s) of the acid phosphatase molecule.

Precipitated acid phosphatase obtained after extensive dialysis against dilute buffer required a solvent having a salt concentration of at least 0.5 M for dissolution (Fig. 1). This material had a 280/260 (nm) absorbancy ratio of 0.90. We took advantage of the solubility properties of the eluted fraction and were able to separate acid phosphatase from other proteins which were more soluble in dilute buffer solution. However, most of the purified material which displayed a 280/260 (nm) absorbancy ratio higher than 1.70 was soluble in dilute buffer (0.05 M Tris-hydrochloride, *p*H 8.5). Thus, it appears that contaminating 260 nm-absorbing material affects the solubility of acid phosphatase by requiring solutions of high salt concentration.

Purified staphylococcal acid phosphatase appeared as a homogeneous protein by gel filtration (second cycle on Sephadex G-100), starch-block electrophoresis, and analytical ultracentrifugation. Attempts to characterize purified enzyme by disc-gel electrophoresis at *p*H 8.3 and 7.5 were unsuccessful because the sample failed to migrate. Purified acid phosphatase migrated toward the cathode of a starch block at *p*H 8.0.

Cutinelli and Galdiero (7) demonstrated that the cell wall of *S. aureus* behaves like a weak ion-exchange resin and that divalent cations combine with the cell wall more readily than monovalent ions. It appears then that the surface of *S. aureus* bears a negative charge as do most bacteria. Since phosphatase was eluted with potassium and sodium ions in the alkaline *p*H range (15), the enzyme may undergo ionic exchange with the cations of the eluting menstruum and therefore would be expected to have basic properties at *p*H 8.0. An extracellular nuclease which is a comparatively small molecule of *S. aureus* was also basic at this *p*H value (11). Acid phosphatase of bovine milk exhibited a positive charge when dispersed in buffer at *p*H 8.6 (3).

Concentration of salt had little effect on

staphylococcal acid phosphatase activity up to 1.0 M KCl or NaCl (Fig. 7). However, progressive loss of enzymatic activity did occur at higher salt concentrations. There was a rather narrow *p*H range (5.2 to 5.3) for optimal activity (Fig. 6). Barnes and Morris (2) also concluded that there is critical dependence on *p*H for optimal activity of staphylococcal acid phosphatase. They found the enzyme from a clinical isolate maximally active at *p*H 5.6. An acid hexose phosphatase from *E. coli* demonstrated optimal activity between *p*H 5.5 and 6.0 (8).

The molecular weight of purified staphylococcal acid phosphatase was estimated to be 58,000. On the other hand, the enzyme of *E. coli* has a molecular weight of 13,000 (12).

The role of staphylococcal acid phosphatase is yet unknown. Further studies of the properties and localization of the enzyme are necessary to permit insight into the biological function of this enzyme.

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