PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 468-472 (1976)

Appearance of Inorganic Pyrophosphatase during Growth of *Streptococcus mutans* Cells[1] (39570)

GEORGE E. CLARK AND VICTOR FRATTALI

Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland 20014; and The George Washington University, Washington, D. C. 20006

Inorganic pyrophosphate (PP_i), which is believed to be bound to the surface of hydroxyapatite crystals, has been suggested as a possible inhibitor of demineralization as well as a regulator of bone formation (1). Isolation and purification of inorganic pyrophosphatase (EC 3.6.1.1, PP_iase) from *Streptococcus mutans*, an organism highly implicated in dental caries (2), are the first phase in a study designed to determine if the enzyme, by hydrolysis of PP_i, is capable of initiating decalcification of enamel and dentin.

Before purifying a bacterial enzyme, two important questions must be answered. At what time during growth of the organism is the enzyme at its maximum level, and what cell disruption technique is appropriate for solubilization of the enzyme in terms of maximum yield? To answer these questions, an apparatus was designed to grow *S. mutans* cells which allowed continuous monitoring of the absorbance of the culture broth and the withdrawal of broth samples for analysis of cell PP_iase levels. Precise cell-harvest time for maximum yield of PP_iase as well as the appearance of PP_iase during growth of *S. mutans*, can be determined from use of the apparatus. Cell disruption techniques were also explored for an efficient method of solubilizing the enzyme.

Methods. Growth of S. mutans cells. *S. mutans* K1R cells were grown in a 4-liter carboy (Fig. 1). Growth medium (4 liters) was 3% Todd-Hewitt broth (BBL, Cockeysville, Md.) and an additional 2% dextrose. The level of inorganic phosphate (Pi) in the broth was 40 mg/100 ml. Todd-Hewitt broth was autoclaved for 30 min, sterile dextrose was added, and all carboy connections were sealed or clamped before removal from the autoclave. Culture medium was inoculated with a 150-ml, 3% Todd-Hewitt, 16-hr culture of K1R cells and incubated at 37° while perfused with a mixture of 90% N_2–10% CO_2 to achieve microaerophilic conditions. A monostaltic pump circulated broth between the carboy and a cuvette tube in a Coleman Junior spectrophotometer (Coleman Electric Co., Maywood, Ill.) for a continuous monitoring of absorbance throughout the growth of K1R cells. Another line was installed from the broth to outside of the carboy to withdraw culture broth samples during the experiments of monitoring the appearance of PP_iase during growth of cells.

Growth of K1R cells was terminated at the height of log phase of growth by centrifugation at 10,000*g*, 20 min, and 4°. Harvested cells were washed three times with 0.05 *mM* Tris-HCl, pH 7.5, and then resuspended, except as noted later, in 10 ml/g of cells (wet weight) of 0.1 *M* Tris-HCl, pH 8.0 (final cell-buffer suspension).

Appearance of PPiase during growth of cells. At periodic times during the growth of cells, 50-ml broth samples, called periodic samples and labeled sequentially A–M, were removed from the culture and cells were immediately harvested by centrifugation at 10,000*g*, 15 min, and 4° (all following centrifugations were in this manner except as noted). Cell pellets from the periodic samples were individually washed three times with 0.05 *M* Tris-HCl, pH 7.5. PP_iase was solubilized from each periodic sample by suspension in 5 ml of 0.1 *M* Tris-HCl, pH 8.0, and ultrasonic treatment (described

[1] Portions of this study were taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This research was supported by the Naval Medical Research and Development Command, Research Task No. MR041.20.02.0318A31J. The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department or the naval service at large.

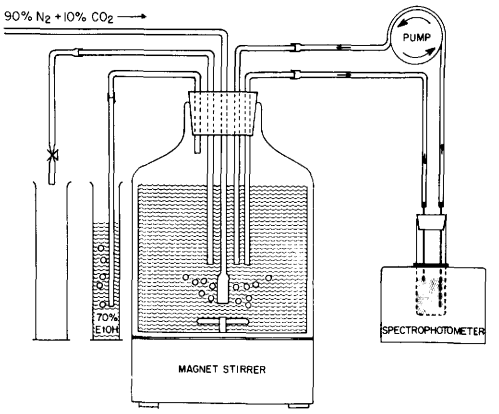

FIG. 1. Apparatus used to grow *S. mutans* K1R cells. A 150-ml, 3% Todd-Hewitt, 16-hr growth of K1R cells was used to inoculate 4 liters of 3% Todd-Hewitt broth, supplemented with 2% dextrose; culture was incubated at 37°. Stoppered line was used to draw off cell-broth samples as desired.

later). The periodic sonicates were also labeled A–M according to their originating periodic sample and assayed for PP_iase activity.

Solubilization of PPᵢase. Five different cell disruption techniques were attempted to solubilize K1R PP_iase from a complete cell harvest from 4 liters of culture growth. Cells were harvested at the height of the log phase of growth on the basis of results from the appearance of PP_iase during the 24-hr growth experiment.

(i) *Braun homogenization.* Twenty milliliters of final cell-buffer suspension was added to 75-ml Duran flasks containing 30 g of glass beads, 0.12-mm diameter. Flasks were shaken for 3 min in a Braun homogenizer (VWR Scientific, Baltimore, Md.) at 4000 cycles/min under CO_2 flow for cooling. Glass beads were removed by filtration over a coarse sintered glass filter. Tris-HCl, 0.1 *M*, pH 8.0, was used to rinse flasks and beads retained by the filter. Unbroken cells and fragments were removed from the filtrate by centrifugation.

(ii) *French press.* Final cell-buffer suspension was added to a precooled French press flask and forced through the flask at 15,000 psi (American Scientific Co., Silver Spring, Md.). Unbroken cells and fragments were removed from the homogenate by centrifugation.

(iii) *Freeze-thaw.* Final buffer suspension was in 50 ml of 0.01 *M* Tris-HCl, pH 8.0. Cell suspension was frozen slowly overnight at −4°. Frozen cell suspension was thawed at room temperature and then centrifuged. Cell suspension was frozen and thawed five successive times in this manner. Supernatants were stored frozen until eventual pooling after the fifth thawing.

(iv) *Osmotic shock.* The cell pellet from the final cell wash was resuspended in 0.033 *M* Tris-HCl, pH 7.2, and osmotically shocked according to an established procedure (3).

(v) *Ultrasonic treatment.* Cells from the final buffer wash were resuspended in 25 ml of 0.2 *M* Tris-HCl, pH 8.0. This final cell suspension was sonified by a Branson sonifier, Model W185 (Heat Systems-Ultrasonics, Inc., Plainview, Long Island, N.Y.), with a flat sonifying tip at 4°, and a power setting between 8 and 9. After 15 min, cell suspension was centrifuged and supernatant pH was adjusted to 7.2 with 0.1 *M* Tris. Unbroken cell pellet was resuspended in 20 ml of 0.1 *M* Tris-HCl, pH 8.0, and sonified again as above. Third and fourth sonications were in 10 and 8 ml, respectively, of the above buffer. Assays for PP_iase activity were accomplished in the individual homogenates and in a pool of homogenates from a 4-liter growth of K1R cells.

Assay of PPᵢase activity. The PP_iase activity in the cell breakage supernatants was determined by modification of an established procedure (4). The assay solution, 1 ml and pH 9.0 (37°), contained 0.8 μmole $Na_4P_2O_7$ (PP_i), 1.6 μmole $MgCl_2$, 50 μmole Tris-maleate-Tris (Sigma Chemical Co., St. Louis, Mo.), and enzyme diluted in 2 μmole Tris buffer. Assay solution was incubated at 37° for 15 min. Enzyme activity was stopped by cooling to 4°. After 1 min of cooling, 2 ml of colorimetric solution of 0.75 *N* H_2SO_4, 0.375% $(NH_4)_6Mo_7 \cdot 4H_2O$, 0.45% $NaHSO_3$, and 0.15% *p*-methylaminophenol sulfate was added to the standard assay solution. After an additional 5 min at 4°, assay-colorimetric solution was incubated at room temperature for 20 min and then the absorbance was determined at 670 nm. Maximum color development due to presence of phosphate, hydrolysis product of PP_i, was obtained by this procedure with insignificant acid hydrolysis of PP_i. Assays were done in

duplicate and a zero time enzyme blank, enzyme added after 15 min at 37°, was included with each duplicate. Known amounts of P_i-buffer tubes were also assayed as reference standards. A unit of PP_iase activity is 1 μmole of P_i released from PP_i per minute at 37°.

Protein determination. Protein concentrations were measured by the method of Lowry *et al.* (5). Bovine serum albumin was used as a reference standard.

Results. Formation of PP_iase during growth of cells. Figure 2 shows the formation of PP_iase activity by K1R cells during 24 hr of growth in Todd-Hewitt broth culture. PP_iase activity increased rapidly during the early phases of growth. The rapid rise in activity was demonstrated by the activity curve coinciding with the growth curve until the height of the exponential phase of growth was reached, whereupon the PP_iase activity fell about as rapidly as it rose during exponential growth. The enzyme activity leveled off at a base line level of activity of approximately 20% of the maximum reached at 0.5 OD units at 660 nm. The base line PP_iase activity level continued throughout the stationary phase of growth. Periodic cell samples A–C required only 1–2 sonications each. Beginning with periodic cell sample D, the K1R cells were more difficult to disrupt. Periodic cell samples E–M required 3–4 sonications for complete cell disruption.

Solubilization of S. mutants PP_iase. Disruption of strain K1R cells by ultrasonic treatment, Braun homogenizer, French press, freeze-thaw, and osmotic shock methods revealed that ultrasonic treatment was the cell disruption technique of choice on the basis of measurable PP_iase activity in the crude homogenates. Table I outlines the results of PP_iase activity yield in the crude homogenates according to the disruption technique. Ultrasonic treatment of harvested K1R cells resulted in a yield of PP_iase activity that was 280% of the PP_iase yield from Braun homogenization of harvested K1R cells. Disruption by the French

TABLE I. DISRUPTION OF *Streptococcus mutans* STRAIN K1R CELLS BY FIVE CELL-DISRUPTION TECHNIQUES.

Cell disruption technique[a]	Units of PPi'ase activity	Protein (mg)	Specific activity
Ultrasonic treatment	4212	1266	3.3
Braun homogenizer	1540	1188	1.3
French press	98	121	0.8
Freeze-thaw	96	248	0.4
Osmotic shock	—	—	—

[a] Each technique was applied to a 4-liter growth in Todd-Hewitt broth; cells were grown to OD of 0.5, at 660 nm.

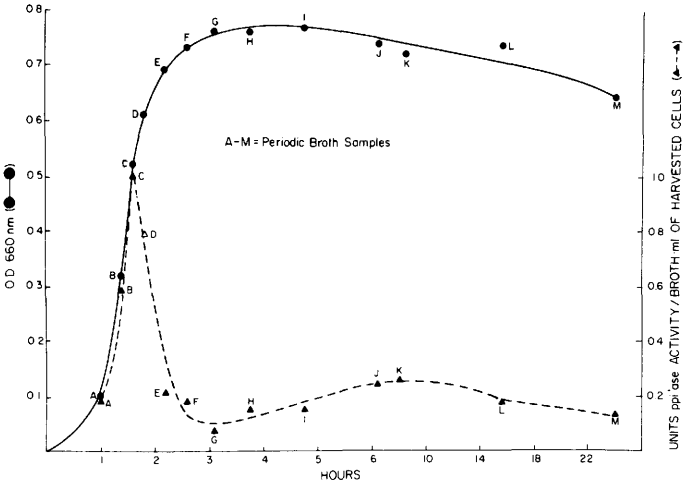

FIG. 2. Appearance of PP_iase activity of *S. mutans* K1R cells during 24 hr of growth. At periodic times, A–M, broth samples were removed from the culture, centrifuged, and sonicated. The resultant periodic sonicates, A–M, were assayed for PP_iase activity.

press and freeze-thaw methods resulted in negligible levels of PP_iase activity. Osmotic shock of K1R cells yielded no detectable PP_iase activity.

Figure 3 depicts typical results of an actual ultrasonic treatment of harvested K1R cells from a 4-liter growth. PP_iase activity was always greatest in the homogenate from ultrasonic treatment when cells are harvested promptly at 0.5 O.D. The pH of the first ultrasonic homogenate was found to drop to as low as 4.8 when the K1R cells grew rapidly or when there was minimal lag phase of growth as shown in Fig. 2. It was also found that the yields of PP_iase activity were always highest when K1R cells grew rapidly with minimal lag phase. Under such conditions, three ultrasonic treatments were always sufficient for yield greater than 95% PP_iase from cell disruption.

Discussion. The finding of the PP_iase activity level rising rapidly to the same extent as the increase in number of strain K1R cells as measured by optical density was similar to the formation of phosphatase ($P_i$ase) by *S. mutans,* strain Ingbritt, cells (6). The $P_i$ase activity of the strain Ingbritt cells remained at a relatively constant level after reaching its maximum level of activity. Contrary to this finding, the PP_iase activity of the strain K1R cells fell about 80% immediately after reaching its highest level at the height of the log phase of growth. This requires that the cells should be harvested at a precise point in the growth curve for maximum yield of PP_iase activity. The rapid increase in PP_iase activity can be explained by the organism producing the enzyme in parallel to the rapid growth of cells. The sudden fall in activity might be explained by curtailment of the production of PP_iase as cell growth is curtailed during the stationary phase of cell growth and the emergence of high levels of an inhibitor such as P_i.

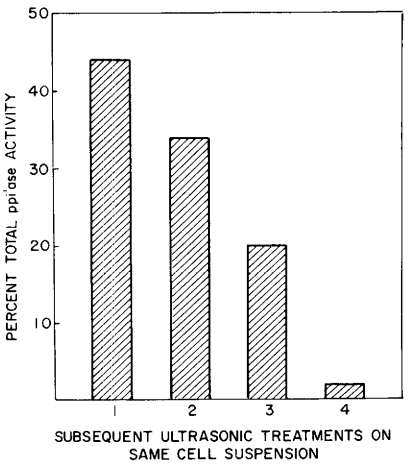

FIG. 3. PP_iase activity in the supernatant of subsequent ultrasonic treatments on the same *S. mutans* K1R cell suspension from a 4-liter culture growth.

High cell levels of P_i, as a product of PP_iase activity and as a result of growth in a phosphate-containing medium during this study, would be expected to inhibit the enzyme. As the cellular P_i level increases, production of additional P_i levels from PP_i may be inhibited by P_i binding to the enzyme molecule. Indeed, studies have shown cariogenic streptococci were able to concentrate intracellular P_i from tooth origin (7). A rapid drop in PP_iase activity on the part of *S. mutans* may be explained in part by the cell's ability to rapidly take in phosphate from teeth and the rest of the plaque environment. Also, the *S. mutans* PP_iase may in some way, yet to be determined, play a role in the cell's ability to tap P_i of teeth and alveolar bone supporting teeth by hydrolysis of PP_i originating from these structures. In so doing, hydroxyapatite of teeth and bone, according to the inhibitory role of PP_i (1), may be susceptible to demineralization as a result of biochemical activities by plaque microorganisms.

A demineralization-remineralization equilibrium has been postulated to take place at a potential carious site (8). During active caries, the equilibrium would shift toward demineralization. Additional recent evidence has suggested that alkaline PP_iase plays a role in the onset of calcification (9). Within the realm of the above postulation and according to the role suggested for alkaline and acid PP_iase (1, 9), *S. mutans* PP_iase may promote remineralization under alkaline conditions and demineralization under acid conditions.

Summary. It is important for the purpose of obtaining PP_iase activity to promptly harvest *S. mutans* K1R cells at the height of the log phase of growth or an OD of 0.5 at 660 nm. The apparatus shown in Fig. 1 enables

one to grow streptococcal cells and to determine specifically during the growth cycle when to harvest the cells. Of the five cell disruption techniques tried, ultrasonic treatment is obviously the technique of choice for disruption of *S. mutans* K1R cells and solubilization of PP_iase for maximum yield.

1. Russell, R. G. G., and Fleisch, H., Clin. Orthop. **69,** 101 (1970).
2. Shklair, I. L., Develop. Ind. Microbiol. **16,** 217 (1975).
3. Nossal, N. G., and Heppel, L. A., J. Biol. Chem. **241,** 3055 (1966).
4. Josse, J., J. Biol. Chem. **241,** 1938 (1966).
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L and Randall, R. J., J. Biol. Chem. **193,** 26 (1951).
6. Knuuttila, M. L. E., and Mäkinen, K. K., Arch Biochem. Biophys. **152,** 685 (1972).
7. Luoma, H., Arch. Oral Biol. **15,** 509 (1970).
8. Poole, D. F. G., and Newman, H. N., Natur (London) **234,** 329 (1971).
9. Anderson, H. C., and Sajdera, S. W., Fed. Proc **35,** 148 (1976).

Received May 25, 1976. P.S.E.B.M. 1976, Vol. 153