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Metabolic Studies with ^{65}Zn. X. Biosynthesis of Alkaline Phosphatase in Cultures of *Pseudomonas fluorescens*

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Alkaline phosphatase from *Pseudomonas fluorescens* has been partially purified. Labelled $^{65}ZnCl_2$ in the culture medium is incorporated in the most purified preparations. The enzyme shows a *p*H optimum of 7.2-7.5 and a K_m value for p-nitrophenyl phosphate of 1×10^{-4}. Zinc ions at high concentration inhibit enzyme activity. The protective effect of Mg on the inactivation of alkaline phosphatase by heat is also reported.

Alkaline phosphatase, is a genuine zinc enzyme (15) formed in the development of various microorganisms (3).

Several investigators have studied the effect of orthophosphates and other compounds on the control of enzyme synthesis. HORIUCCHI (7) and TORRIANI (14) have found, that the presence of orthophosphates in the culture medium of *Escherichia coli* repress alkaline phosphatase synthesis. GAREN and LEVINTHAL (4) have observed that the percentaje of enzyme recovered during purification increases when the levels of inorganic phosphatase decreases in the enzyme preparation.

In *Bacillus subtilis* grown in a phosphate-rich medium, MOSES (13) has found an increase in activity after transferring cells grown in the phosphate-rich medium to a medium containing low concentration of phosphate. Similar results have been observed in *Neurospora crassa* (9) and *Bacillus licheniformis* (8).

More recently, GHOSH *et al.* (5) have discovered a mutant of *Bacillus subtilis* strain SB-15, designated SB-1004 in which alkaline phosphatase synthesis, seems to be independent of the phosphate content in the medium, sugesting that enzyme formation can also be constitutive, as in a case previously described by DORN (1) in *Aspergillus nidulans*. This author, had interpreted his finding as due to the existence of two isoenzymes, I and II the

former being inducible and the latter constitutive.

In the present report, we have studied the incorporation of ^{65}Zn into the prosthetic group of alkaline phosphatase from *Pseudomonas fluorescens* and the effect of different substances on enzyme formation and activity.

Materials and Methods

Organism: Pseudomonas fluorescens strain CECT-378 has been used throughout this work.

Media composition: Several complex and synthetic media were employed. Nutrient broth (Difco), Osawa's medium and Levinthal's medium produced high yields of enzyme and a modification of the latter which produced highest levels of enzyme was finally adopted (12). Final peptone concentration in the culture was 2 g/l, and the medium was supplemented ocassionally with $^{65}ZnCl_2$ to reach a 2×10^{-6} M concentration of Zn^{++} ions in the culture.

Alkaline phosphatase activity: Enzyme was determined following the method of DORN (2) modified as described before (11). p-nitrophenyl phosphate was used as substrate and the amount of p-nitrophenol liberated by hydrolisis was measured by the absorbance at 400 nm in a spectrophotometer Unicam S.P.200. Units of enzyme are international units.

Protein concentration was determined following LOWRY's method (10).

Results

Figure 1 shows the production of alkaline phosphatase and growth of *Pseudomonas fluorescens* strain CECT-378 in different media. It was observed that growth on Nutrient broth gives a high yield of organisms but low enzyme amounts was produced during growth on this medium. In contrast, growth on a modification (12) of Levinthal's medium was slower and less amount of cells were produced during a similar incubation perior. However, high levels of enzyme were synthesized during growth on this medium. Growth and enzyme formation in Osawa's medium was lower than in Nutrient broth of Levinthal's.

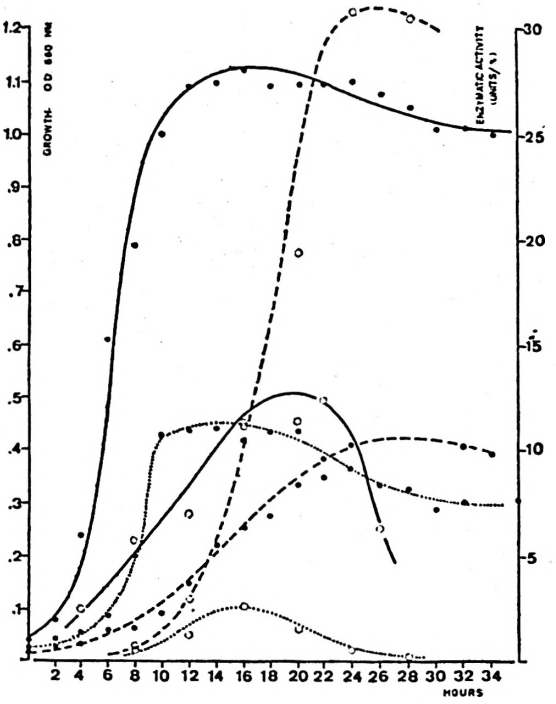

Fig. 1. *Relation between Growth* (●) *and enzymatic activity from alkaline phosphatase* (○) *with differents culture mediums.* Osawa (···), Levinthal (---) and Nutrient broth (—).

In order to study the effect of Zn^{++} ions on alkaline phosphatase, synthesis, it was considered important working with a partially purified enzyme preparation of known properties. To this end, an 18 h. culture of *Pseudomonas fluorescens* in Levinthal's medium modified were harvested by centrifugation at 5,000 × g for 20 min., and resuspended in 0.01 M veronal buffer, *p*H 7.4 (12). No enzyme activity could be detected in the supernatant which was therefore discarbed. Cell extracts were

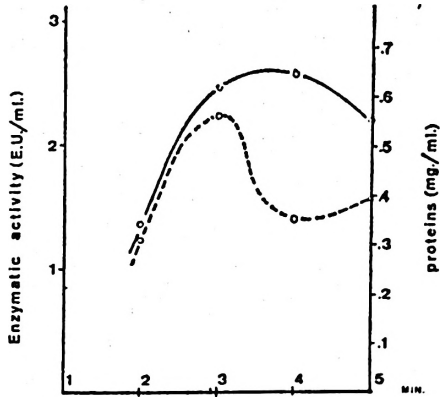

Fig. 2. *Alkaline phosphatase liberation: Ultrasonic optima time desintegration (prot. —), (enzymatic activity - - -).*

obtained by sonnication and as it is shown in figure 2 sonnication for 3 min. gave highest enzyme yields. Cell debris was separated by further centrifugation at 20,000 × g for 5 min. and the clear supernatant was designated fraction I.

Ammonium sulphate precipitation: Solid ammonium sulphate was added to fraction I and the suspension formed was stirred for 30 min. at 4° C to obtain saturations between 10 % and 90 %. At each step, the precipitate was removed by centrifugation at 10,000 × g and the supernatant used again. When 90 % saturation was reached no detectable amounts of alkaline phosphatase remained in the supernatant.

Precipitates obtained in the interval 15-50 % saturation, were resuspended in 0.01 M veronal buffer *p*H 7.4, pooled, and dyalised aigainst 5 l of the same buffer for 24 h. Non dyalizable material was considered as fraction II.

Gel-filtration through Sephadex G-100: Aliquots of fraction II were applied to a column of «Sephadex G-100» (5 × 80 cm) equilibrated with 0.01 M veronal buffer *p*H 7.4. The column was eluted with the same buffer and 5 ml fractions were collected. Enzyme activity and protein concentration was determined in all fractions (fig. 3). In experiments in which $ZnCl_2$ (^{65}Zn) had been added to the medium radioactivity was also measured in each fractions.

Characterization of alkaline phosphatase. a) *Optimum pH:* Enzyme activity in the partially purified preparation from

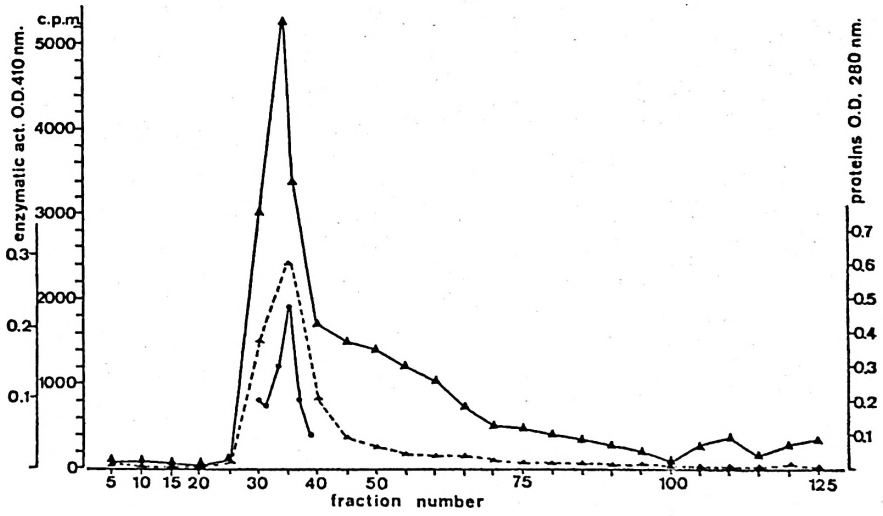

Fig. 3. *«Sephadex G-100» Gel filtration (Counts per minute ▲—▲, proteins ▲---▲, enzymatic activity ●—●).*

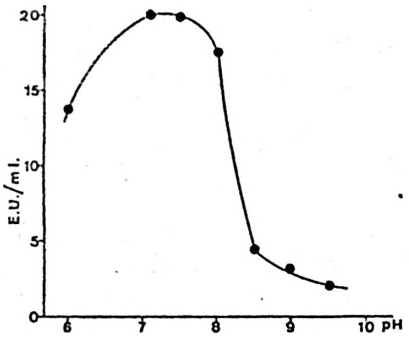

Fig. 4. *Alkaline phosphatase activity: pH effect.*

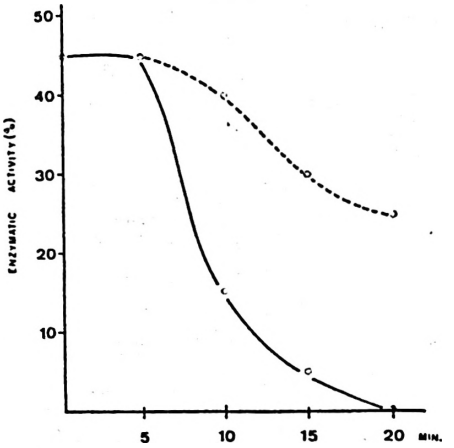

Fig. 5. *Heat inactivation of alkaline phosphatase: Mg^{++} protector effect (Control —, $MgCl_2$ 10^{-2} M ---).*

«Sephadex G-100» was determined in 0.01 M veronal buffer at a range of *p*H of 6 to 9.5. As shown in figure 4, *p*H optimum in this buffer lies between 7.2-7.5.

b) *Stability:* Inactivation of alkaline phosphatase from *Pseudomonas fluorescens* by heat takes place quickly at 80° C (fig. 5). As shown in this figure addition of $MgCl_2$ (1 mM) protects to some extent the enzyme from inactivation during a period of 15 min. After this time, the rate of inactivation is similar to that of a control.

c) *Effect of substrate concentration:* Kinetics of alkaline phosphatase of *Pseu-*

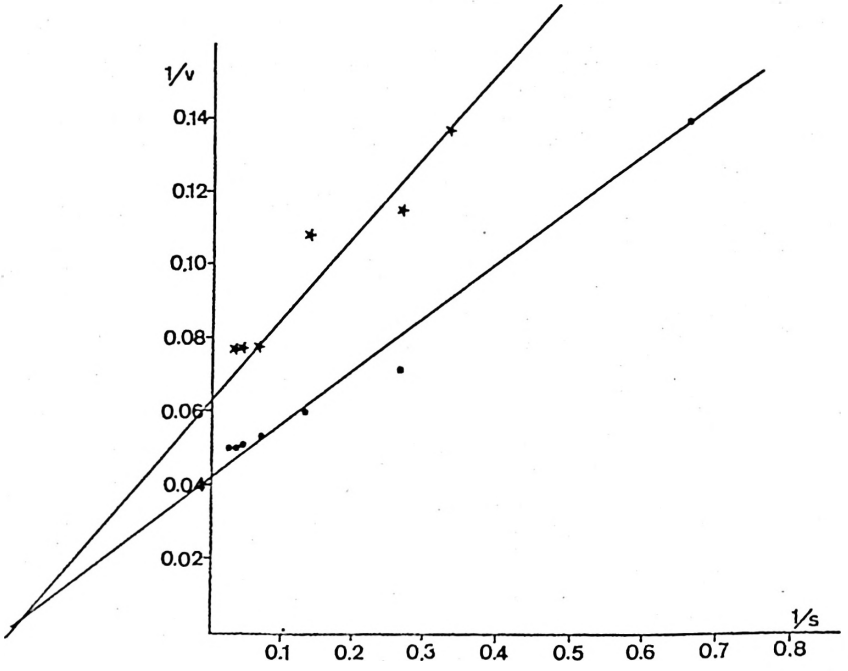

Fig. 6. *Alkaline phosphatase inhibition produced by Zinc (no Zn ✳—✳; 1×10^{-4} M $ZnCl_2$ ★—★).*

domonas fluorescens show typical hyperbolic curves. To determine the affinity for the synthetic substrate used, the initial velocity of enzyme activity was determined at different substrate concentration and plotted according to the method of LINEAWER and BURK. K_m value obtained from these plots (fig. 6) was 1×10^{-4} M. In the figure is also shown the inhibitory effect of zinc iones.

Incorporation of ^{65}Zn into alkaline phosphatase: To determine wether radioactivity from ^{65}Zn is incorporated into alkaline phosphatase, cells of *Pseudomonas fluorescens* were incubated in Levinthal's medium containing $^{65}ZnCl_2$ and enzyme was extracted and purified as described. As shown in figures 3 and 7, the peak of radioactivity incorporated closely parallels the bands containing the higher activity of alkaline phosphatase both in ammonium sulphate fractionation and in gel filtration, suggesting that ^{65}Zn is preferentially incorporated into the prosthetic group of the enzyme.

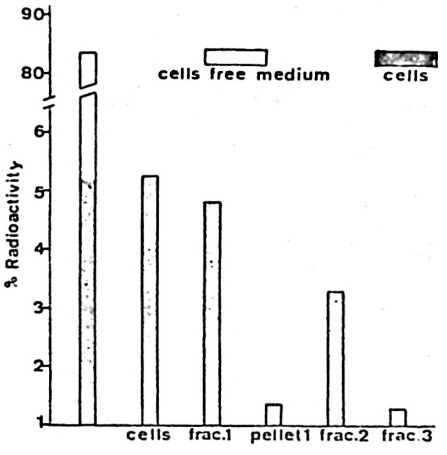

Fig. 7. *Distribution of radioactivity during alkaline phosphatase purification steps.*

Discussion

In accordance with the values reported for the properties of alkaline phosphatase from several sources (4, 14), the enzyme from *Pseudomonas fluorescens* is repressed by inorganic phosphates. Thus as shown in figure 1 the enzyme activity in a medium such as Levinthal's modified which is lacking inorganic phosphate, is high, while in Osawa's medium activity is practically absent.

Addition of ^{65}Zn to the medium, leads to incorporation of radioactivity into the protein fraction that contains alkaline phosphatase separated by two different procedures. Thus, in the eluate of the Sephadex column and during precipitation with ammonium sulphate fractions showing activity of phosphatase also had high activity ^{65}Zn. The remaining radioactivity was however, heterogenously distributed (figs. 3 and 7).

As to the properties of the enzyme from *Pseudomonas* it has been calculated that the optimum *p*H in the presence of p-nitrophenyl phosphate used as a substrate, for the activity of the alkaline phosphatase produced by this strain of *Pseudomonas fluorescens*, is between 7.2 and 7.5 (fig. 4). When *p*H increases above these values, activity drops off until practically becoming nil at a *p*H of 9.5. If compared with data obtained for the same enzyme in other microorganisms, its behavior resembles that of the alkaline phosphatase isolated by GAREN and LEVINTHAL (4) from *Escherichia coli* whose optimum *p*H is 8, since that isolated by TORRIANI (14) for the same enzyme and microorganisms gives a *p*H of between 8.8 and 8.9; the optimum *p*H for the alkaline phosphatase isolated from the *Neurospora crassa* is found by KADNER (9) to be between 9 and 9.5.

According to GAREN *et al.* (4) the alkaline phosphatase from *Escherichia coli* is a very thermostable enzyme, since it retains its activity when incubated at 85° C for 30 minutes and magnesium seems to protect the stability of the above mentioned enzyme from the action of heat (6). DORN (2) states that the alkaline

phosphatase isolated from *Aspergillus nidulans* becames inactive when heated to 65-75° C for 30 minutes; and HULETT-COWLING (8) asserts that this enzyme isolated from the *Bacillus licheniformis* does not became denatured ven if kept at 80° C for five minutes.

The results obtained with regard to the thermostability of the enzyme from *Pseudomonas fluorescens* at 70° C and at different times, show that above this temperature the enzyme is totally inactivated, In agreement with the results of other authors, the Mg^{++} ion also acts as a moderate stabilizer.

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