Regulation of the Formation of Acid Phosphatases by Inorganic Phosphate in Aspergillus ficuum

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Two types of extracellular acid phosphatases are synthesized by Aspergillus ficuum NRRL 3135: a nonspecific orthophosphoric monoester phosphohydrolase (EC 3.1.3.2) with an optimum pH of 2.0, and an enzyme with restricted specificity, a mesoinositol-hexaphosphosphate phosphohydrolase (EC 3.1.3.8; phytase) with an optimum pH of 5.5. Although the pH 5.5 enzyme is termed a phytase, both enzymes hydrolyze phytin. Synthesis of the enzymes is repressed by high orthophosphate concentrations in the fermentation medium. The highest total level for each enzyme is synthesized in low orthophosphate medium. In high orthophosphate medium, more pH 5.5 enzyme is produced than pH 2.0 enzyme. In low orthophosphate medium, more pH 5.5 enzyme is produced than pH 2.0 enzyme during the early stages of growth, but the reverse occurs after 5 days. The enzymes are differentiated by heat denaturation at acid and alkaline pH levels. They are separated into two distinct fractions on Sephadex G-100 followed by carboxymethylcellulose column chromatography. This indicates that the two enzymes are structurally different. The $K_m$ for both enzymes is 1.25 mm when calcium phytate is the substrate. Orthophosphate competitively inhibits the pH 2.0 ($K_i = 1.1 \times 10^{-3}$ M) but not the pH 5.5 phytase.

Neither enzyme is denatured by 50% (w/v) urea or inhibited by 0.01 M tartrate. Thus, they differ from human prostatic phytase.

The properties of various types of acid phosphonooesterases obtained from various sources have been described and classified (9). We have been interested in the type of acid phosphatase which hydrolyzes phytate to inositol and inorganic orthophosphate (P$_i$; reference 10; J. H. Ware and T. R. Shieh, U.S. Pat. No. 3,297,548). Phytases present in plants and microorganisms have been reviewed (11).

Although certain enzymes have been called phytases, they are fairly nonspecific. They hydrolyze some of the organic phosphates more rapidly than they do phytin. Wheat germ phytase is a good example (7).

The control of acid phosphatase synthesis by the concentration of inorganic phosphate in the medium has been reported for the extracellular enzymes of Aspergillus ficuum (10; Ware and Shieh, U.S. Pat. No. 3,297,548) and A. awamori var. kawachii (8). The type and amount of phophatase synthesized is dependent on the concentration of inorganic phosphate in the medium (1–3, 8).

It was demonstrated that 0.02 M tartrate inhibited acid phosphatases from the following sources: human prostate, mouse prostate, kidney, liver, cardiac and skeletal muscle, and tumors (5). Although the human prostate contains two acid phosphatases which can be separated by chromatography, both react in essentially the same manner toward various substrates and inhibitors (12).

This report describes the regulation, separation, substrate specificity, and some properties of two distinct acid orthophosphonomonoesterases produced by A. ficuum. The studies in this paper lead to the following conclusions. (i) A. ficuum produces a relatively specific extracellular phosphatase (phytase) which hydrolyzes phytic acid at a faster rate than any of the substrates tested at pH 5.5. (ii) The regulation of the acid phosphatase is controlled by the concentration of inorganic phosphate in the medium. (iii) The phytase differs from other phosphatases previously reported.

MATERIALS AND METHODS

Organism. Spores of A. ficuum NRRL 3135 were formed and maintained in 100-ml screw-top dilution bottles containing 25 ml of Malt Yeast Extract Agar (Difco) at pH 9.0 and 28 C. A spore suspension

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containing 10⁶ to 2 × 10⁷ spores/ml was made from the above with 0.1% (w/v) sodium laurate. A portion (0.5 ml) of this spore suspension was used to inoculate 50 ml of autoclaved fermentation medium described in Fig. 1 of Shieh and Ware (10). The same medium was used throughout, except that the inorganic phosphate calculated as phosphorus (Pᵢ) was varied where indicated. The cultures were incubated on a rotary shaker (270 rev/min) at 28 C.

Materials. All chemicals were reagent grade unless otherwise specified. The various substrates were from Nutritional Biochemicals Corp., Cleveland, Ohio. Calcium phytate was purified free of Pᵢ (10).

Buffers. The 0.2 m buffers for the pH optimum experiments were as follows: hydrochloride-water (pH 1 to 1.5), glycine-hydrochloride (pH 2 to 3.5), sodium acetate-chloride (pH 4.0 to 5.5), sodium succinate-chloride (pH 4.5 to 6.0) and imidazole-chloride (pH 6.3 to 7.6).

Measurement of enzyme activity. Phosphatase activity was assayed by following the release of orthophosphate. The reaction was initiated by adding 0.1 ml of suitably diluted enzyme preparation to 0.9 ml of each of the 0.2 m buffers containing 2.5 μmole of purified calcium phytate. The reaction mixture was incubated for 5 to 20 min at 37.5 C. Liberated Pᵢ was determined by the method of Fiske and SubbaRow (4). One phosphatase unit was defined as the amount of enzyme that liberates 1 μmole of Pᵢ/min at 37.5 C. Phosphatase activity was proportional to time of incubation and enzyme concentration during the incubation period.

Separation of the acid phosphatases. Cell-free culture filtrate (1,260 ml) was concentrated by lyophilization, suspended to 380 ml with water, and dialyzed overnight in cellophane at 28 C. The dialyzed sample was applied to a column of Sephadex (8 by 40 cm; G-100 Pharmacia) previously equilibrated with 0.05 M NaCl. The enzymes were eluted with 0.1 M NaCl by use of a Gilson Medical Electronics drop fraction collector; 10-ml fractions were collected. Two entities (I and II) were partially separated on Sephadex G-100.

The material in the tubes representing fraction II was combined and lyophilized. The resultant 30 mg of material was dissolved in 50 ml of water and dialyzed overnight in cellophane against running water at 28 C. The dialyzed sample was applied to a column of carboxymethylcellulose (1.5 by 50 cm; BioRad cellex-CM) previously equilibrated with 0.05 M glycine-chloride buffer (pH 3.0). After adsorption of the protein, the column was washed with 50 ml of water.

Elution was stepwise with 30 ml each of glycine-hydrochloride (pH 8.5) buffer of the following concentrations in the order used: 0.05, 0.10, 0.15, 0.20, 0.30, and 0.40 M. Fractions (2.5 ml) were collected as above.

RESULTS

Effect of pH on acid phosphatase activity. The crude phosphatase in the culture filtrate of A. ficuum had two pH optima (Fig. 1) when propagated in the low (2 mg of Pᵢ/100 ml)-phosphate medium described by Shieh and Ware (10). When phytate was used as the substrate, the pH optima were at 2.0 and 5.5. The pH optima were the same (Fig. 2) when the organism was propagated in high (20 mg of Pᵢ/100 ml)-phosphate medium. However, the relative amounts of pH 2.0 and pH 5.5 activity differed and were dependent on the Pᵢ concentration in the culture medium.

Differential denaturation of the acid phosphatases by heat. The phosphatase activity with a pH optimum of 5.5 was completely denatured by heat (70 C) in 5 min at pH 3.0 (Fig. 1), whereas only 25% of the pH 2.0 enzyme was denatured under the same conditions. If the heat denaturation was done at pH 8.5, 65% of the pH 2.0 enzyme was

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**FIG. 1.** Effect of pH and heat on acid phosphatase synthesized by Aspergillus ficuum in low-Pᵢ (2 mg/100 ml) medium. The substrate was calcium phytate; the conditions of assay as were described. The enzyme activity at pH 2.0 is equal to 100% relative activity.

**FIG. 2.** Effect of pH on acid phosphatase synthesized by Aspergillus ficuum in high-Pᵢ (20 mg/100 ml) medium. Conditions of assay as in Fig. 1. The enzyme activity at pH 5.5 is equal to 100% relative activity.
specificity denatured. Therefore, the pH 2.0 enzyme is relatively stable to heat at pH 3.0, and the pH 5.5 enzyme is stable to heat at pH 8.5.

Substrate specificities of the acid phosphatases. The specificity of the crude phosphatases was tested against 20 different phosphate ester substrates at pH 2.0 and at pH 5.5 (Table 1). The reaction rate was slower at pH 5.5 than at pH 2.0 for all of the substrates tested. Galactose-6-phosphate, 6-phosphogluconic acid, 3-glycerophosphate, propanediolphosphate, carbamyl phosphate, DL-0-phosphoserine and phosvitin were not hydrolyzed at pH 5.5 but were hydrolyzed at pH 2.0. The order of relative rate of hydrolysis of the substrates differed and was dependent on the pH of the reaction. One of the substrates, phytic acid, was hydrolyzed at about the same rate at either pH level. Although some of the other substrates were hydrolyzed slowly at pH 5.5, the only substrate which exhibited two distinct pH optima was phytic acid.

Effect of Pi in the medium and time on the synthesis of acid phosphatases. High (20 mg/100 ml) concentrations of Pi in the medium repress the synthesis of the pH 2.0 and the pH 5.5 acid phosphatases (Fig. 3). However, the ratio of the pH 2.0 enzyme to the pH 5.5 enzyme is less in high Pi medium than in low Pi medium. Higher concentrations of the pH 5.5 enzyme are present early in the fermentation. As the phosphorus concentration in the medium is increased, the ratio of the pH 2.0 to pH 5.5 phosphatase decreases (Fig. 4). Highest concentrations of both the pH 2.0 and pH 5.5 enzymes are synthesized in low Pi medium. Therefore, both enzymes are repressed by the presence of Pi.

Separation of the acid phosphatases. The crude culture filtrate of *A. ficuum* containing the acid phosphatases was dialyzed, lyophilized, and subjected to molecular sieve chromatography on Sephadex G-100. Two peaks, I and II, were distinguishable but not adequately separated (Fig. 5). Peak I contained a high proportion of the pH 2.0 phosphatase and peak II contained a high proportion of the pH 5.5 phosphatase. The fractions comprising peak II were combined and lyophilized. The resultant 50 mg of material was dialyzed and subjected to carboxymethylcellulose chromatography. Two fractions were separated (Fig. 6). The material eluted first contained the acid phosphatase which was active only at pH 2.0. The slow-moving material had activity at both pH 2.0 and 5.5. This is not surprising, since the pH 5.5 enzyme has a broad pH optimum and has some activity at pH 2.0.

Initial velocity patterns for saturation by substrate. Typical Lineweaver-Burk plots (6) for the

| Table 1. Substrate specificity of *Aspergillus ficuum* phosphatases at pH 2.0 and pH 5.5a |
|--------------------------------------|----------|----------|
| Substrates b                         | pH 2.0   | pH 5.5   |
|                                     | Units/ml | Relative Activity (%) | Units/ml | Relative Activity (%) |
| p-Nitrophenyl-phosphate             | 8.40     | 100      | 0.10     | 1.2               |
| Glucose-6-phosphate                 | 8.20     | 97       | 0.46     | 5.5               |
| Galactose-6-phosphate               | 4.80     | 57       | 0.22     | 2.6               |
| Fructose-6-phosphate                | 7.20     | 86       | 0.22     | 2.6               |
| Fructose-Diphosphate                | 6.40     | 76       | 0.46     | 5.5               |
| Galactose-6-phosphate               | 3.60     | 43       | 0        | 0                 |
| Inositol-monophosphate              | 3.00     | 36       | 0.46     | 5.5               |
| 6-Phosphogluconic acid              | 5.40     | 64       | 0        | 0                 |
| Ribose-5-phosphate                  | 3.60     | 43       | 0.22     | 2.6               |
| Adenosine-triphosphate              | 5.40     | 64       | 0.10     | 1.2               |
| Riboflavin-phosphate                | 6.20     | 74       | 0.46     | 5.5               |
| b-Glycerophosphate                  | 5.20     | 62       | 0        | 0                 |
| Dl-Glycereraldehyde 3-phosphate     | 5.00     | 59       | 0.22     | 2.6               |
| Phosphoglyceric acid                | 4.40     | 52       | 0.10     | 1.2               |
| Propandiol-phosphate                | 2.20     | 26       | 0        | 0                 |
| Carbamylphosphate                   | 2.00     | 24       | 0        | 0                 |
| dl-O-Phosphoserine                  | 1.00     | 12       | 0        | 0                 |
| Phosvitin                           | 4.20     | 50       | 0        | 0                 |
| Phytic acid                         | 2.20     | 26       | 1.60     | 18                |

a Enzyme is the culture filtrate from low Pi (2 mg/100 ml) medium.
b Reaction mixtures contained 2.5 μmoles of phosphorus as Pi; glycine-hydrochloride and succinic-hydrochloride buffers were used at pH 2.0 and 5.5, respectively.
c Units = μmoles of Pi/min as described.
d Relative activity at pH 2.0 with p-nitrophenyl-phosphate as the substrate is defined as 100%.

separated pH 2.0 and pH 5.5 phosphatases of *A. ficuum* in the presence and absence of 3 × 10⁻³ M Pi are shown in Fig. 7. The Kₘ for both enzymes is 1.25 mM when calcium phytate is used as the substrate. Pi does not inhibit the pH 5.5 phytase but it does competitively inhibit the nonspecific pH 2.0 phosphatase. The Kᵢ is 1.1 × 10⁻³ M. The activity of the pH 2.0 and pH 5.5 enzymes was not altered by 50% (w/v) urea or 0.01 M tartrate.

**DISCUSSION**

This report provides evidence for the synthesis of two distinct extracellular acid orthophosho-
The enzymes are differentiated by their pH optima, substrate specificity, resistance to denaturation by heat at various pH levels, separation by chromatography, inhibition by Pi, and synthesis regulation by Pi. These data preclude the possibility that they are isozymes.

The total amount of phosphatases synthesized is dependent upon the initial Pi concentration in the medium; and high Pi levels repress acid phosphatase synthesis (10). The concentration of Pi in the medium not only controls the total amount of acid phosphatase synthesized but it also controls which particular acid phosphatase is synthesized (Fig. 3 and 4). Our studies agree with those of Ohta et al. (8) who described differences in phosphatase levels of five chromatographic peaks whose concentration and appearance was dependent on the Pi in the medium and the age of the culture. Since the concentration of Pi affects the ratio of the two phosphatases synthesized and since these phosphatases are not isozymes, different genes must be responsible for the synthesis of the pH 2.0 and the pH 5.5 enzyme by A. ficuum.

The enzyme with a pH 5.5 optimum described...
in this report is the only phosphatase tested with a number of organic phosphates which hydrolyzes phytate at a faster rate than any of the common substrates usually tested (Table 1). The phytase described in this report might be useful as an analytical enzyme for the determination of phytate phosphorus in natural products.

LITERATURE CITED