A Repressible Alkaline Phosphatase in Neurospora crassa*

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SUMMARY

The discovery was made that an orthophosphate-repressible alkaline phosphatase exists in Neurospora crassa. Variable phosphate levels in the culture medium caused the enzyme to vary in the order of 100-fold in its range of specific activities. The properties of this 20-fold purified enzyme clearly differentiated it from the alkaline phosphomonoesterase of N. crassa, a P1-repressible alkaline phosphomonoesterase in Escherichia coli, and the alkaline phosphatase of yeast. The enzyme is a nonspecific phosphohydrolase with esters and also pyrophosphate. It is stimulated by ethylenediaminetetraacetate and unaffected by a number of metallic ions tested in the absence of ethylenediaminetetraacetate. The enzyme is stable in the pH range between 7 and 8 but loses activity rapidly at pH 9.0. This loss of activity at pH 9.0 is retarded by phosphate esters serving as substrates, by certain metallic ions, and by P1. The presence of both a repressible and a P1-nonrepressible alkaline phosphoesterase in N. crassa represents a finding not generally observed in other species.

EXPERIMENTAL PROCEDURE

Growth of Cultures—Normal strain 1A of N. crassa was used in all experiments. The basic phosphate-free growth medium, described in a previous communication (4), was supplemented with the required amounts of potassium phosphate. All of the cultures were grown at 25° and harvested 3 days after conidial inoculation. The large amounts of vegetative growth needed for purification of the enzyme came from 4-liter aerated cultures containing 50 µmoles of P1 per liter of medium. The studies concerning the effect of phosphate on enzyme concentration in mycelium were done with 100-ml surface cultures grown in Roux flasks. No alkaline phosphatase activity was released into the culture medium by the mycelium during growth.

Enzyme Assay—Alkaline phosphatase activity was routinely assayed by incubating the enzyme for 10 min at 37° with a 5 mM solution of the substrate p-nitrophenyl phosphate in 0.3 M glycine buffer at pH 9.0, the pH optimum for the reaction. The incubation mixture also contained 1 µmole per ml of EDTA. The p-nitrophenol released by the enzyme was determined by the method of Bessey, Lowry, and Brock (5). At the substrate level used, the release of phosphate by a given amount of enzyme at 37° was proportional to the duration of the assay for the first 30 min of incubation. A unit of enzyme activity was defined as the quantity of enzyme that catalyzes the release of 1 µmole of P1 per hour at these specific conditions. Specific activity was defined as the enzyme units per mg of protein. Since either 1 mM EDTA or the 0.3 M glycine buffer used in the assay medium prevents the enzymatic expression of the previously described alkaline phosphomonoesterase (2), this procedure could be used to determine the repressible alkaline phosphatase in crude extracts containing both of these alkaline esterases. The acid phosphomonoesterase (1) does not interfere with the assay because it is inactive at pH 9.0. This fortuitous specificity of the assay mixture made possible the determination of the repressible enzyme in crude extracts from mycelium grown on varying phosphate levels in the medium. With the exception of these repression studies (Fig. 1), all of the other enzyme determinations were made on alkaline phosphatase that had been separated from the acid and alkaline phosphomonoesters by column chromatography. With the exception of p-nitrophenyl phosphate, all of the other substrates were assayed by incubating for 25 min under the prescribed conditions, and the P1 released was determined colorimetrically by the method of Fiske and SubbaRow (6). Protein was routinely measured by the colorimetric procedure of Lowry et al. (7). Bovine albumin was used as a reference standard for protein determinations.

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The previously described acid and alkaline phosphomonoesterases were assayed in fractions obtained after column chromatography (Fig. 2) by procedures based on the original methods of Kuo and Blumenthal (1, 2). The incubation mixture for assaying the acid phosphatase was 0.3 M acetate buffer, pH 5.6, and for assaying the alkaline phosphomonoesterase it was 0.3 M Tris buffer, pH 8.9, with 1 mM MgCl₂. Enzyme incubations were for 25 min at 37°C with 10 mM β-glycerophosphate as the substrate. A unit of activity for these enzymes was defined the same as for the P₁-repressible phosphatase.

Preparation of Crude Extracts—Unless otherwise stated, all of the procedures involving the extraction and purification of the enzyme were done at 2–4°C and deionized distilled water was used.

The P₁-repression studies requiring the quantitative extraction of enzyme from tissues were done with duplicate cultures grown concurrently under identical conditions on different levels of Pi in the medium. The mycelium from each culture was harvested in a Servall Omni-Mixer with the blades turning at 5,000 rpm for 15 min at 12,000 X g after standing for 1 min. The supernatant was immediately dehydrated by centrifugation for 10 min at 12,000 X g. The residue resulting after decanting the supernatant was resuspended in about 5 parts of water and reprecipitated by centrifugation as before. The combined extract and water wash contained 80 to 85% of the total alkaline phosphatase. Most of the measurable enzyme retained by the residue was released in a solution by incubating a suspension of this particulate fraction for 16 hours at 4°C in 15 parts of an aqueous solution containing 0.50% deoxycholate in 0.02 M glycylglycine buffer, pH 8.0. The homogenate was centrifuged for 15 min at 12,000 X g in a type SS-1 Servall centrifuge. The residue resulting after centrifugation was resuspended in about 5 parts of water and reprecipitated by centrifugation as before. The combined extract and water wash contained 80 to 85% of the total alkaline phosphatase.

Preparation of crude extracts from 2 g (dry weight) of mycelium, usually in a volume of about 10 ml, was put on a DEAE-cellulose column that was identical with that employed by Kuo and Blumenthal (1) for the purification of the acid phosphomonoesterase from N. crassa. The enzyme elutions were accomplished by first eluting the column with 100 ml of 0.02 M acetate buffer, pH 5.6, followed by an increasing concentration gradient of acetate buffer at pH 5.6 according to the procedure of Kuo and Blumenthal (1). The rate of elution was 30 ml per hour, and 10-ml samples of the eluate were collected and analyzed for the presence of phosphatase activity and protein. The concentration of buffer in the eluates was determined by conductivity measurements with a Radiometer type CDM2 meter. The elution pattern for a typical column is shown in Fig. 2. The repressible alkaline phosphatase is eluted near the solvent front of the initial 0.02 M acetate buffer (pH 5.6) while the acid and alkaline phosphomonoesterases remain on the column until a higher concentration of buffer at the same pH is passed through the column. This chromatographic procedure gave a 3- to 4-fold purified enzyme (Table I), free of the two other esterases, and it also separated the desired phosphatase from substances which interfere with its reproducible precipitation by ammonium sulfate in the subsequent purification step.

Removal of Inactive Proteins by 80% Saturation with Ammonium Sulfate—The column fractions containing the alkaline phosphatase were combined and lyophilized to give a solution containing between 140 and 170 units per ml. To this was added an equal volume of a concentrated buffer (pH 7.2) which was 0.5 M with respect to both maleic acid and Tris. The resulting buffer solution was brought to 80% saturation at 0°C with ammonium sulfate (325 mg per ml), allowed to stand 1 hour, and centrifuged for 10 min at 12,000 X g. The supernatant was immediately decanted along with any flocculent material that remained either suspended or at the surface of the solution, and additional ammonium sulfate was added to bring this cloudy solution to 90% saturation at 0°C (88 mg per ml in addition to the original amount of ammonium sulfate at 80% saturation). Once again, the mixture was centrifuged 10 min at 12,000 X g after standing for 1 hour at 0°C. The alkaline phosphatase was in the insoluble fraction that was recovered after 90% saturation with ammonium sulfate. If the supernatant at this step of purification appeared cloudy after centrifugation, additional enzyme could often be obtained by filtering the supernatant on a diatomaceous filter aid from which the enzyme could readily be recovered by washing with water or dilute buffer at neutral pH. The total enzyme recovered from this procedure was routinely used in these studies. Its specific activity of about 1,000 units per mg of protein represented a 20-fold purification based on the activity observed in crude extracts of mycelium (Table I).

Materials—The glucose 6-phosphate, galactose 6-phosphate, 2-phosphoglyceric acid, and phosphoenolpyruvic acid were from Sigma. The DEAE-cellulose was a product of the Bio-Rad Laboratories with an exchange capacity of 0.70 meq per g. All of the other organic reagents were obtained from Calbiochem.

RESULTS

The data in Fig. 1 show the effect of variable P₁ levels in the growth medium on the concentration of a herebefore unknown alkaline phosphatase in the mycelium of N. crassa harvested during a period of rapid growth (3-day cultures). These determinations could be made on crude extracts because the medium used for enzyme assay was designed to negate competition for substrates by the two other known phosphatases in the mold (1, 2). When the enzyme-deficient extracts from cultures grown on media containing high P₁ concentrations were subjected to DEAE-cellulose column chromatography, the resulting eluates were also deficient in phosphatase activity in those column fractions where the repressible alkaline phosphatase would be expected (Fig. 2). Extracts of P₁-repressed cultures failed to show appreciable enzyme activity in the standard assay when tested with a variety of substrates. Precautions were taken to make...
The repressible alkaline phosphatase was eluted by 0.02 M acetate buffer in the top reservoir. The enzyme elutions was possible to do studies on the activity of the enzyme in the presence of Pi. However, there was a very rapid loss of enzyme (60 to 70% loss in 1 min at 37°) at pH 9.0 where it is at its optimal activity.

The crude water extracts of mycelium from cultures grown on medium initially containing 50 μM P_i per liter yielded a 20-fold purified enzyme after DEAE-cellulose chromatography and ammonium sulfate precipitation (Table I). The effect of pH on the activity of this repressible phosphatase is presented in Fig. 3. The phosphatase exhibited activity over a wide pH range with maximum activity observed at pH 0.0 to 0.5. The enzyme had some activity on the acid side of neutrality, in contrast to the previously described alkaline phosphomonoesterase of N. crassa (2), which is active only in the alkaline range. The samples of the enzyme at various stages of purification all showed essentially the same relative changes in activity with changes in pH. In the alkaline pH range, the phosphatase showed better activity in glycine buffer than in Tris buffer (Fig. 3). Since 1 mM EDTA enhanced the activity of the enzyme at all stages of purification, it was routinely added to the incubation media.

The enzyme could be stored at −20° for several months at pH 7 to 8 without loss of activity. In this pH range, it did not lose measurable activity after 1 hour at 37° or after 24 hours at 4°; however, there was a very rapid loss of enzyme (60 to 70% loss in 1 min at 37°) at pH 0.0 where it is at its optimal activity. It was possible to do studies on the activity of the enzyme in the upper pH range because the rapid deterioration of the enzyme above pH 8 was prevented by phosphate esters which serve as substrates. At the substrate levels used in most of these studies (1 to 20 mM), the enzyme exhibited negligible loss of activity during the first 30 min of incubation. The reaction rate remained a linear function of the enzyme concentration in the range of enzyme levels employed (between 0.1 and 1.0 unit of enzyme per ml of incubation mixture). The activity of the alkaline phosphatase at its pH optimum was stimulated by EDTA and was unaffected by Mg++, Zn++, Mn++, Fe++, Fe++++, Co++, Cu++, or Cu+++ at concentrations up to 1 mM. The rapid loss of alkaline phosphatase

![Graph](image-url)
activity in the absence of substrate at pH 9.0 was greatly retarded by Ca++, Mn++, and Zn++. For example, in the presence of 0.1 mM Zn++, the enzyme retained 80% of its original activity after incubation for 15 min in 0.3 M glycine buffer, pH 9.0, at 37°C.

The repressible alkaline phosphatase from N. crassa was found to be markedly inhibited by Pi. The kinetics of this inhibition is of the competitive type (Fig. 4), that is, Pi increases the $K_m$ for p-nitrophenyl phosphate without changing the maximal velocity. From the data in Fig. 4, the $K_m$ for p-nitrophenyl phosphate was calculated to be $3.3 \times 10^{-4}$ M, and the $K_i$ for Pi, $2.5 \times 10^{-4}$ M.

The substrate specificity of the alkaline phosphatase preparation is shown in Table II. In addition to its general phosphomonoesterase activity, the enzyme has some pyrophosphatase activity. Although it hydrolyzes PPi and releases some Pi from NADPH, it does not show measurable activity toward either FAD or L-α-glycerophosphorylcholine. The 20-fold purified enzyme was tested against combinations of two substrates to determine if the properties of the phosphatase preparation might actually be the result of a summation of the properties of two or more closely related phosphoesterases. Two rapidly hydrolyzed substrates, β-glycerophosphate and p-nitrophenyl phosphate, and PPi, a less rapidly hydrolyzed substrate, were used in combinations to see if there were some additive effects on their rate of hydrolysis due to activity by more than one enzyme. Table III contains the results of an experiment in which the rates of hydrolysis of these three substrates, individually and in pairs, were determined. These data indicated that the amount of Pi formed corresponded closely with the amount expected from a single enzyme acting simultaneously on two substrates. The calculations were based on the formulas of Thorn (9) and Whittaker and Adams (10).

**DISCUSSION**

A 20-fold purified alkaline phosphatase was obtained from N. crassa. The concentration of the enzyme in fast-growing my-
The presence in N. crassa of two alkaline phosphomonoesters, one of which is product repressed, represents a unique situation which has not been generally observed in other species. This observation suggests that some interdependent relationship between the two enzymes may play a regulatory role in the turnover of phosphate esters in the cells. In N. crassa, the range of specific activities over which an enzyme can be caused to vary, generally of the order of 10-fold, is much less than that encountered in bacteria (14). For example, the formation of aspartate carbamoyltransferase is limited to a repression-depression variation of 2-to 3-fold in N. crassa (15), in contrast to the 1000-fold variation found in E. coli for the same enzyme (16). Other repression-depression variations noted in N. crassa were 2-fold variation for nitrate reductase (17), 2-fold in a normal strain for δ-carboxy-δ-hydroxyisocaproic acid isomerase (18), 2-fold for pyrroline-5-carboxylate reductase (19), 5-fold for ornithine transcarbamylase (20), and 4-fold for carbamylphosphokinase (20). The range of specific activities over which enzymes can vary in N. crassa is sometimes greater in mutant strains than in the normal strains (18, 21). Large variations in the specific activity of N. crassa tyrosinase have been noted by Horowitz, Fling, MacLeod, and Sueoka (22) and Horowitz, Fling, and Asano (23). Aromatic amino acids are effective as inducers for this enzyme, and sulfate, or a substance derived from it, acts as a repressor of the synthesis. Both tyrosinase and L-amino acid oxidase, normally undetectable during vegetative growth, can be simultaneously derepressed by starvation after a lag of 9 to 12 hours, or both enzymes can be induced by certain inhibitors of protein synthesis in a medium which otherwise does not allow synthesis of these enzymes (23).

The magnitude of the repression-depression variation noted here for the alkaline phosphatase is therefore unusual in this mold. The success of future studies pertaining to the control mechanisms involved in the synthesis of this repressible alkaline phosphatase will be dependent on the availability of mutant strains bearing aberrations involving the production of this enzyme. Studies are in progress to develop a selective screening method for finding such mutant strains.

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