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AN ALKALINE PHOSPHOMONOESTERASE FROM *NEUROSPORA CRASSA*

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SUMMARY

An alkaline phosphomonoesterase was purified 40-fold from mycelium of *Neurospora crassa*. The enzyme had a pH maximum of 8.9–9.0 with β-glycerol phosphate as substrate and exhibited maximal activity in the presence of Mg^{2+}. The enzyme was nearly completely resolved with respect to its Mg^{2+} requirement and was very sensitive to inhibition with Be^{2+}. The substrate specificity of the enzyme was studied using 21 compounds and the properties of the enzyme were compared with those of the acid phosphomonoesterase previously isolated from the same organism.

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INTRODUCTION

Alkaline phosphomonoesterases have been recognized in various fungi on a number of occasions although relatively little is known of their properties[1]. There have been reports of an alkaline phosphomonoesterase in extracts of *Neurospora crassa*[2,3] and a few of its properties were ascertained because of its presence as a contaminant in preparations of an L-histidinol phosphate phosphatase. During the course of a study on the acid phosphomonoesterase of *N. crassa*[4], an alkaline phosphomonoesterase was detected in the crude mycelial extracts. This afforded an opportunity to compare the properties of the acid phosphomonoesterase with those of an alkaline phosphomonoesterase formed under identical conditions.

EXPERIMENTAL

Materials

The sources of the substrates and reagents used in these studies were the same as those previously described[4]. Mycelia of *N. crassa*, strain 5297a (wild-type) was grown, harvested and stored in the same manner. The alkaline phosphomonoesterase was completely stable in the frozen mycelia for over a year.

Enzyme assay

β-Glycerol phosphate was routinely used as the substrate. The reaction mixture (0.65 ml) contained the following (in μmoles): β-glycerol phosphate, pH 8.9, 10; sodium barbital–sodium carbonate buffer, pH 8.9, 30; magnesium sulfate, 5; and appropriate amounts of enzyme. The enzymic reaction was initiated by addition of the enzyme solution to the temperature-equilibrated assay tubes and after 20 min incubation at 30°, the reaction was stopped by addition of 0.1 ml of 10% trichloroacetic acid. Any precipitate formed was removed by centrifugation and the supernatant fluid was used for the determination of P_i. The routine control was a complete

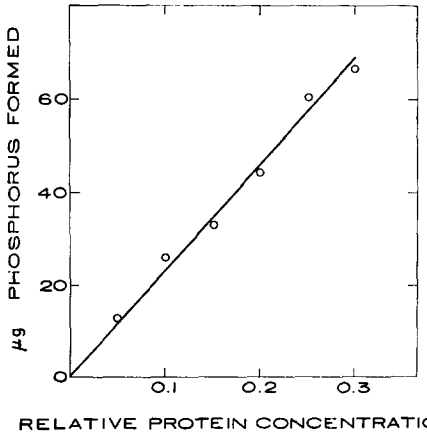

Fig. 1. Reaction rate as a function of enzyme concentration. The reaction mixtures contained samples of diluted enzyme, purified through step 3, 10 μmoles of sodium β-glycerol phosphate, pH 8.9, 30 μmoles of sodium barbital–sodium carbonate buffer, pH 8.9 and 5 μmoles of magnesium sulfate in a final volume of 0.65 ml.

assay tube to which the trichloroacetic acid had been added at zero time. When acid-labile substrates were used in the assay, the control tube contained substrate, buffer and boiled enzyme. At the end of the 20-min incubation period, the trichloroacetic acid was added and the P_i assayed by the method of PEEL AND LOUGHMAN[5]. The release of P_i by a given amount of enzyme was proportional to the duration of the assay and the proportionality of P_i released with increasing amounts of enzyme is shown in Fig. 1. A unit of enzyme activity was defined as the quantity of enzyme that would catalyze the liberation of one μmole of phosphorus/min under the conditions specified. Specific activity is defined as the enzyme units/mg of protein.

Analytical methods

P_i was determined colorimetrically by either the method of FISKE AND SUBBAROW[6] or the method of PEEL AND LOUGHMAN[5]. The latter method, modified to maintain the pH at 4.0 by a 4-fold increase in the amount of acetate buffer, was used to assay P_i in the presence of acid-labile substrates. Protein was routinely measured by the procedure of WADDEL[7] except in chromatographic fractions where the method of WARBURG AND CHRISTIAN[8] was used.

Purification of enzyme

Unless otherwise specified, all operations were carried out in a cold room at 2–4° and deionized, glass distilled water was routinely used.

Preparation of crude extract (step 1): Frozen mycelia (100 g), 200 g of washed glass beads and 400 ml of water were minced for 5 min in a Waring blender. Cell debris and beads were removed by centrifugation for 10 min at 12000 × *g*. The supernatant fluid usually had approximately equal quantities of acid[4] and alkaline phosphomonoesterases.

Fractionation with ammonium sulfate (step 2): Finely-powdered ammonium sulfate (112 g) was added while stirring to 400 ml of crude extract (0.4 saturation). After standing for 1 h in an ice-bath, the precipitate that was removed by centrifugation for 20 min at 15000 × *g* was discarded. An additional 56 g of ammonium sulfate were added (0.6 saturation) and after standing for 1 h, the precipitate was collected by centrifugation. The precipitate was dissolved in 10 ml of 0.01 *M* Tris-buffer, pH 8.8, and dialyzed for 6 h against 2 l of 0.01 *M* Tris, pH 8.8, containing 0.02 *M* KCl. The acid phosphomonoesterase, which is soluble in 0.6 saturated ammonium sulfate[4], was now separated from the alkaline phosphomonoesterase.

Fractionation on a DEAE-cellulose column (step 3): DEAE-cellulose, that had been washed with 1% NaOH in 10% NaCl, 10% NaCl and 0.01 *M* Tris, pH 8.8, in that order, was used to pack a column 18.7 × 160 mm. After the column was equilibrated with 0.02 *M* KCl in 0.01 *M* Tris, pH 8.8, the dialyzed enzyme from the ammonium sulfate fractionation was placed on the column. The column was treated successively with 200-ml quantities of 0.02, 0.08, 0.10, 0.12 and 0.14 *M* KCl. Nine-millilitre fractions were collected with a fraction collector and protein and enzyme assays were performed on the fractions. The alkaline phosphomonoesterase was eluted as a broad fraction in tubes 52 through 70 with most of the enzyme in tubes 53–60. These latter fractions were combined, concentrated to a small volume by lyophilization and then dialyzed against 0.01 *M* KCl for 6 h. The dialyzed con-

centrated enzyme could be stored at —20° for several months with no loss in enzyme activity. A summary of the data on purification of the enzyme is presented in Table I.

TABLE I

PURIFICATION OF *N. crassa* ALKALINE PHOSPHOMONOESTERASE

Fractionation Step	*Total units*	*Yield (%)*	*Specific activity (units/mg protein)*
1. Crude extract*	106	100	0.047
2. $(NH_4)_2SO_4$, 40–60% saturation; dialyze; lyophilize	51	48	0.205
3. DEAE-cellulose chromatography; lyophilize; dialyze	48	43	2.051

* In this purification procedure, 100 g of frozen mycelia were processed.

RESULTS

Properties of alkaline phosphomonoesterase

The properties of the enzyme were determined with enzyme purified through step 3, unless otherwise noted.

Influence of pH

The results of experiments on the influence of pH on the enzyme activity are presented in Fig. 2. The maximum activity, with β-glycerol phosphate as substrate, was observed at pH 8.9–9.0 in sodium barbital–carbonate, sodium barbital–acetate, Tris–HCl or glycine–NaOH buffers. Borate buffer was strongly inhibitory for the enzyme while imidazole buffer was less inhibitory. The absence of any substrate hydrolysis at pH 5.6 indicated that acid phosphomonoesterase[4] was absent from the enzyme preparation.

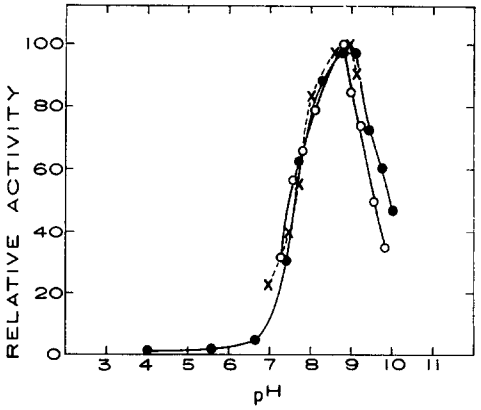

Fig. 2. The influence of the variation of pH on the liberation of phosphorus from β-glycerol phosphate. The enzyme activities are indicated as the percentage of the activity at pH 8.9 in the barbital–carbonate buffer. The standard assay described in the text was performed except for the variation in type and/or pH of the buffer. The pH shown is the actual pH at the completion of the incubation, as measured with a glass electrode on a duplicate sample. O—O, barbital–carbonate buffer; ●—●, barbital–acetate buffer; ×—×, Tris buffer.

The enzyme was more stable under alkaline conditions than at acidic conditions. The crude extract lost only 3 % of its activity when kept at pH 9.0 for 5 h at 30° whereas at pH 4.0, 5.6 or 8.4 it lost 82, 58 and 34 %, respectively, of its alkaline phosphatase activity.

Influence of cations

In order to study the divalent cation requirements of the enzyme, a 2-ml sample of enzyme solution that had been purified through step 3 was further dialyzed for 12 h against two 1000-ml changes of deionized glass distilled water. This enzyme was still not completely resolved with regard to its divalent cation requirement although in the absence of added Mg^{2+}, it had only 38 % of the activity it had when supplemented with the optimal amount of Mg^{2+}. This dialyzed enzyme preparation was used to study the effects of added cations on the enzyme activity (Fig. 3). Mg^{2+} was the most effective cation for restoring enzyme activity while Co^{2+}, Mn^{2+} and Zn^{2+} followed in decreasing order of effectiveness. In the range of ionic concentrations tested, high concentrations of all cations, except Mg^{2+}, were inhibitory. Ca^{2+} and Fe^{2+} were not stimulatory at all the concentrations tested while Be^{2+} was extremely toxic and Cu^{2+} less toxic

TABLE II

SUBSTRATE SPECIFICITY OF *N. crassa* ALKALINE PHOSPHATASE

The enzyme assay was performed as described in the text except that the final concentration of substrate, adjusted to pH 8.9, is as indicated in the table.

Substrate	*Substrate concentration (M)*	*Relative rate of hydrolysis**	*Michaelis constant (M)*	*Relative rate of hydrolysis, acid phosphatase***
β-Glycerol phosphate	0.03	100	$5.9 \cdot 10^{-3}$	94
α-Glycerol phosphate	0.03	85		100
Acetyl phosphate***	0.005	0		56
Phosphoenol pyruvate§	0.008	15		10
α-Glucose 1-phosphate	0.03	8		23
Glucose 2-phosphate	0.03	10		11
Glucose 3-phosphate	0.03	11		13
Glucose 6-phosphate	0.03	40		45
Glucosamine 6-phosphate	0.03	31		89
Ribose 5-phosphate	0.03	32		28
Carbamyl phosphate§§	0.03	23		7
Potassium phosphoramidate§§	0.03	0		28
Adenosine 5'-phosphate	0.03	34		30
O-Phospho-D,L-serine	0.03	0		0
O-Phospho-D,L-threonine	0.03	0		0
L-Histidinol phosphate	0.015	40	$9.1 \cdot 10^{-4}$	7
O-Phospho-L-homoserine	0.008	28		5
p-Nitrophenyl phosphate§§§	0.015	39	$2.2 \cdot 10^{-2}$	51
Potassium pyrophosphate	0.015	0		0
Thiamine pyrophosphate	0.015	0		0
Phosphovitin	0.015	0		0

* The amount of P liberated from β-glycerol phosphate, 1.31 μmoles, was considered to be 100 and the other values are indicated as percentage of this value.

** Values for the relative rate of P liberation from various substrates by the *N. crassa* acid phosphomonoesterase were selected from data in a previous study[4].

*** Acetyl phosphate was determined by the method of LIPMANN AND TUTTLE[9].

§ Phosphoenol pyruvate was added as the cyclohexylamine salt.

§§ Evolution of ammonia was measured in a Conway diffusion vessel[10].

§§§ *p*-Nitrophenol was determined by the method of BESSEY *et al.*[11].

(Table III). Cu^{2+} ($1.5 \cdot 10^{-5}$ *M*) was previously observed not to affect the alkaline phosphatase activity in crude extracts of *N. crassa*[3].

When the enzyme preparation that had been dialyzed against distilled water was stored at —20° for 120 h and reassayed, it was observed that the enzyme still displayed the same activity in the presence of Mg^{2+} although it was now almost completely resolved with regard to its Mg^{2+} requirement. In the absence of added Mg^{2+}, this aged, dialyzed preparation now had only 6 % of the maximal alkaline phosphatase activity observed in the presence of Mg^{2+}.

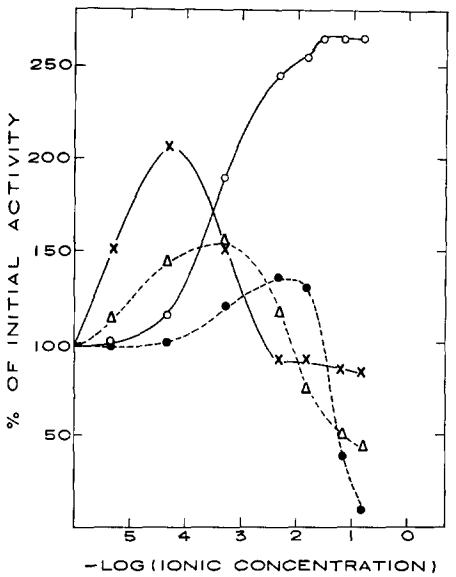

Fig. 3. Effect of cations on the hydrolysis of β-glycerol phosphate by *Neurospora* alkaline phosphomonoesterase. Enzyme purified through step 3 was further dialyzed for 12 h against two 1-l changes of deionized glass-distilled water and was immediately assayed. The regular assay procedure was employed except that 0.05 ml of the type and concentration of the cations used is indicated. Final concentrations are 1/13th of those shown. The results are presented as percentages of the amount of P liberated by the enzyme in the absence of any added divalent cation. O—O, $MgSO_4$; ●—●, $ZnSO_4$; △—△, $MnSO_4$; ×—×, $CoCl_2$.

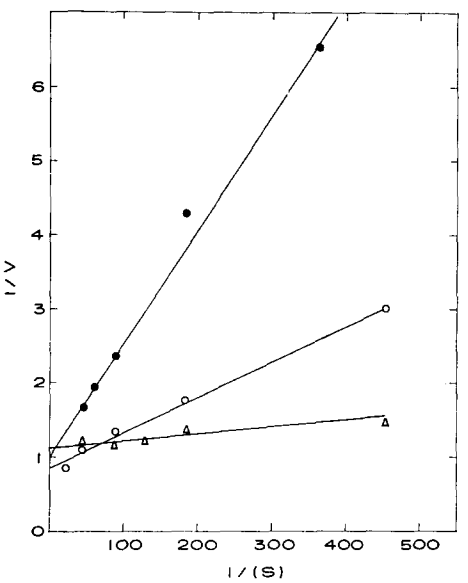

Fig. 4. Effect of substrate concentration, (*S*), on the rate of hydrolysis, *V*, by *Neurospora* alkaline phosphomonoesterase. The results are plotted by the method of LINEWEAVER AND BURK[12]. O — O, β-glycerol phosphate, K_m $5.9 \cdot 10^{-3}$ *M*; ● — ●, *p*-nitrophenyl phosphate, K_m $2.2 \cdot 10^{-2}$ *M*; △ — △, L-histidinol phosphate, K_m $4.1 \cdot 10^{-4}$ *M*.

Substrate specificity

Twenty-one compounds were tested as substrates for the enzyme under the conditions of the standard assay at pH 8.9, except for the concentration of substrate (Table II). Since all of these substrate concentrations were not known to be at levels which would insure maximal rates of hydrolysis, the relative rates of hydrolysis should be considered very approximate. Some, but not all, phosphomonoester bonds were hydrolyzed but inorganic or organic pyrophosphate and phosphoprotein bonds were not cleaved. Of the substrates tested, β-glycerol phosphate was hydrolyzed most rapidly and the same concentration of α-glycerol phosphate was hydrolyzed at 85 % of its rate. Some hydrolysis of histidinol phosphate was probably due to contamination

of the enzyme preparation with histidinol phosphate phosphatase[2]. Thus, when 0.0767 M histidinol phosphate and 0.00031 M β-glycerol phosphate were added together as mixed substrates, the observed rate of P formation was 0.645 whereas the calculated rate[20], based on the rates with the same concentrations of substrates used separately, should have been only 0.536 if only a single enzyme was responsible for the hydrolysis of both substrates. Further evidence for the presence of histidinol phosphate phosphatase, which is insensitive to Be^{2+}, was provided by measuring the hydrolysis of histidinol phosphate in the presence or absence of $8.3 \cdot 10^{-4}$ M Be^{2+}. In the presence of Be^{2+}, histidinol phosphate was cleaved at 10% of the rate achieved in its absence indicating that the Be^{2+}-sensitive alkaline phosphomonoesterase (Table III) was contaminated with a maximum of 10% histidinol phosphate phosphatase.

Influence of substrate concentration

The effect of substrate concentration on the velocity of hydrolysis of three substrates was determined under conditions of constant pH. The LINEWEAVER-BURK[12] plots of $1/V$ *versus* $1/S$ for the substrates are presented in Fig. 4. The K_m values were: β-glycerol phosphate, $5.9 \cdot 10^{-3}$ M; p-nitrophenyl phosphate, $2.2 \cdot 10^{-2}$ M; and L-histidinol phosphate, $9.1 \cdot 10^{-4}$ M.

Effect of inhibitors

Several compounds were tested as inhibitors of the alkaline phosphomonoesterase (Table III). (+) Tartrate, which was a competitive inhibitor for the *Neurospora* acid phosphomonoesterase[4], had no effect on the alkaline phosphomonoesterase while NaF was only a weak inhibitor. The sensitivity of the enzyme to KCN was in agreement with the results demonstrating a cation requirement.

Physical properties of the enzyme

The ultraviolet absorption spectrum of the enzyme showed only a single absorption maximum at 275 mμ with no peak or shoulder near 260 mμ. The enzyme was less stable to heat than was the corresponding acid phosphomonoesterase[4]. Thus samples

TABLE III

EFFECT OF VARIOUS INHIBITORS ON *N. crassa* ALKALINE PHOSPHOMONOESTERASE

Assay medium (0.65 ml) contained 30 μmoles of sodium barbital–sodium carbonate buffer, pH 8.9, 10 μmoles β-glycerol phosphate, pH 8.9, 5 μmoles magnesium sulfate, enzyme and neutralized inhibitor. The tubes, minus substrate, were pre-incubated at 30° for 10 min before addition of the substrate to initiate the reaction. The incubation was at 30° for 20 min.

Inhibitor	*Concentration (M)*	% *Inhibition*
(+) Tartrate	$1 \cdot 10^{-2}$	0
NaF	$1 \cdot 10^{-2}$	7
KCN	$1.3 \cdot 10^{-3}$	70
$BeSO_4$	$1 \cdot 10^{-5}$	99
$BeSO_4$	$1 \cdot 10^{-6}$	22
$CuSO_4$	$2 \cdot 10^{-4}$	64
$CuSO_4$	$2 \cdot 10^{-3}$	100
p-Chloromercuriphenyl sulfonate	$1 \cdot 10^{-4}$	50

of the enzyme, at pH 9.0, were completely inactivated after heating at 60° for 10 min whereas the acid phosphomonoesterase still retained about one-fourth of its activity after heating for 15 min at 60°.

DISCUSSION

The properties of the *N. crassa* phosphatase indicate that it can best be classified as a relatively non-specific, alkaline phosphomonoesterase most closely fitting Roche's classification of a Type I phosphomonoesterase[13]. This is based on the pH optimum of 8.9–9.0, a requirement of Mg^{2+} for maximal activity and the ability to hydrolyze phosphomonoesters.

The properties of this partially purified *N. crassa* alkaline phosphomonoesterase clearly differentiate if from those of the 1400-fold purified acid phosphomonoesterase derived from mycelium grown under identical conditions[4] as well as the alkaline phosphomonoesterase of *Escherichia coli*[14,15] and the alkaline phosphatase of yeast[16].

The highly purified *E. coli* alkaline phosphomonoesterase has a pH maximum of 8.0 (see ref. 14), and can be completely resolved with regard to its Zn^{2+} requirement[17]. The Fe^{2+}-dependent yeast alkaline phosphatase is probably not a phosphomonoesterase since it only hydrolyzes *p*-nitrophenylphosphate and phosphoramidate but not typical phosphomonoesters[16]. *Aspergillus flavus*, however, possesses a Be^{2+}-sensitive, Mg^{2+}-activated alkaline phosphomonoesterase with a pH optimum of 8.75 (see ref. 18) that is similar to the *N. crassa* alkaline phosphomonoesterase; not enough of its properties have been reported to permit a direct comparison of the enzymes. In other reports concerned with microbial alkaline phosphatases[1,13], the description of the enzymes are either not complete enough to permit comparison, or like the *E. coli* and yeast enzymes, differences in the pH optima, cation requirements or other properties allow the conclusion to be made that they are different from the *Neurospora* alkaline phosphomonoesterase.

The substrate specificity of the *Neurospora* alkaline phosphomonoesterase is similar, in some respects, to that of the acid phosphomonoesterase derived from the same mycelium[4] (Table II). Both enzymes will hydrolyze the glycerol and glucose phosphates as well as adenosine 5′-phosphate, ribose 5-phosphate and *p*-nitrophenylphosphate at similar relative rates. Furthermore, both enzymes will not hydrolyze phosphothreonine, phosphoserine or phosphovitin. Some important differences, though, are the lack of hydrolysis of either acetyl phosphate or phosphoramidate by the alkaline enzyme along with an approximately 6-fold increase in the relative rate of hydrolysis of histidinol phosphate and O-phosphohomoserine.

Since the *Neurospora* alkaline phosphomonoesterase is unable to hydrolyze phosphothreonine or phosphoserine, substrates actively cleaved by non-specific animal alkaline phosphomonoesterases[19], a structure such as $HOOC–CHNH_2–CH_2OPO_3H_2$ cannot serve as a substrate for the enzyme. Removal of the carboxyl group or addition of a methylene group between the carbon atoms to which the NH_2 and phosphomonoester groups are attached, such as in histidinol phosphate and phosphohomoserine, respectively, restores the ability of the substrate to serve as a substrate. The specificity of the alkaline phosphomonoesterase for these particular substrates, then, is identical qualitatively with the specificity of the acid phosphomonoesterase.

AMES prepared a 13-fold purified L-histidinol phosphate phosphatase from *N. crassa* mycelium and found the enzyme preparation to be contaminated with an alkaline phosphomonoesterase[2]. He could distinguish the two enzymes, however, because the former was insensitive to 10^{-4} *M* Be^{2+} while the latter was selectively inhibited. From the few properties of the contaminating alkaline phosphomonoesterase reported[2], it appears that it is identical with the alkaline phosphomonoesterase described here.

The possibility that the *Neurospora* acid phosphomonoesterase may play a role in regulating certain cellular metabolic processes has already been discussed[4]. This possibility also applies to the alkaline phosphomonoesterase. Furthermore, the formation of alkaline phosphomonoesterase, like acid phosphomonoesterase, is not repressed by the presence of inorganic phosphate in the growth medium during growth of mycelium[21].

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