Some comparative studies of two alkaline phosphatases from *Aspergillus niger*

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Summary. Alkaline phosphatases (ALP I and ALP II), isolated and purified from *Aspergillus niger* exhibited broad specificity towards a wide variety of substrates, and consistently ALP II was more active than ALP I. It is possible that the difference in levels of activity of the 2 enzymes may be of physiological importance in the mycelia of *A. niger.*

The term, alkaline phosphatase (E.C.3.1.3.1) refers to a group of enzymes sharing the capacity to hydrolyze phosphate esters in an alkaline medium. The subject has been reviewed comprehensively before[2]. Alkaline phosphatase (ALP) isolated from *Aspergillus niger*[3] was found to separate into 2 forms (ALP I and ALP II) during purification. The 2 phosphatases had the same K_m but different V_{max} values towards a non-physiological substrate, p-nitrophenyl phosphate (pNPP). The observed pH optima of the 2 forms were different and ALP I had a higher mol. wt than ALP II[3]. The presence of inorganic phosphate in the medium did not seem to repress the production of these phosphatases. It was not clear to us whether the phosphatases are 2 distinct enzymes or 2 fractions of the same enzyme, separated by the purification produce. Therefore it was deemed necessary to study the activity of the phosphatases towards a variety of physiological substrates and the possible effect of other cofactors on enzyme activity.

Materials and methods. Growth of *Aspergillus niger.* Mycelia were grown and harvested as described elsewhere[3]. Preparation of cell-free extract: about 5 g of frozen mycelia were thawed and suspended in 20 ml of ice-cold 0.01 M-Tris-HCl buffer, pH 8.0 and then homogenized for 2 min at 0 °C. The homogenate obtained was centrifuged at 5000 × g for 20 min at 0 °C. The supernatant obtained was retained for enzyme purification. The procedure adopted for the purification of the 2 forms of alkaline phosphatase (ALP I and ALP II) was essentially the same as described previously[3].

Enzyme assays. Alkaline phosphatase. The procedure of Torriani[4] was followed with respect to pNPP as substrate. The reaction mixture (2.0 ml) contained 200 µl of the respective alkaline phosphatase (ALP I or ALP II), 100 µl of 54 mM substrate (p-NPP) in 2.0 mM-Mg Cl_2 solution and 1.7 ml of 0.2 M Tris-HCl buffer (pH 9.5 for ALP I and pH 8.5 for ALP II). The reaction mixture for ALP I was incubated at 20 °C while that for ALP II was incubated at 30 °C for 10 min. The reaction was stopped by adding 2 ml of 10% (w/v) trichloroacetic acid solution. The precipitate formed was centrifuged off. A fraction (2 ml) of the resultant supernatant was added to 1.0 ml of 1.0 M Na_2Co_3 and the amount of p-nitrophenol liberated was measured spectrophotometrically at 400 nm. One unit of activity is the amount of enzyme causing the release of 1 µmole of p-nitrophenol/min at 20 °C (ALP I) and at 30 °C (ALP II). The level of activity (1.5 IU) was equated as 100% activity for all experiments.

With respect to other phosphorylated substrates, enzyme activity was measured by the release of orthophosphate, following the procedure described by Ames[5] using phosphate buffer as standard. This procedure measures the amount of orthophosphate released. Reaction mixtures routinely contained 50 nM substrate, 250 µl of 0.05 M-Tris-HCl buffer (pH 9.5 for ALP I or pH 8.5 for ALP II) and were initiated by the addition of 200 µl of the appropriate enzyme fraction (ALP I or ALP II). Reactions were terminated by boiling for 1 min. Blank incubation mixtures had no enzyme fraction. The change in absorbance at 820 nm was measured spectrophotometrically.

Ribonuclease activity of the purified fractions was measured using a modification of the procedure of Ishikawa et al.[6]. Routinely, incubation mixtures contained 1 mg RNA in 1.0 ml of 0.05 M-Tris-HCl buffer (pH 9.5 or pH 8.5) and the reaction was started by adding 200 µl of the appropriate enzyme fraction to make a total volume of 1.2 ml. Blank mixtures had no enzyme fraction. Reaction mixtures were incubated at the appropriate temperature (30 °C or 20 °C) and then stopped by the addition of 100 µl of 15% (w/v) perchloric acid. After cooling in ice for 10 min, the mixtures were clarified by centrifugation and a 200-µl sample was diluted 10-fold and the increase in absorbance determined spectrophotometrically at 260 nm.

Deoxyribonuclease activity of purified fractions was determined by a modification of the procedure of Kunitz[7]. An amount (50 mg) of DNA was suspended in 50 ml of 0.05 M Tris-HCl buffer (pH 9.5 or pH 8.5) and left overnight at 5 °C. A sample (5 ml) of the DNA solution was added to 200 µl of the appropriate enzyme fraction, incubated at the appropriate temperature (30 °C or 20 °C) and the increase in absorbance at 260 nm was compared to that of a blank (no enzyme added). For the denatured DNA (dnDNA), a sample (5 ml) of the DNA solution (as above) was heated at 100 °C for 15 min and cooled quickly before the addition of the enzyme.

Results. The 2 fractions of alkaline phosphatase (ALP I and ALP II) purified by the procedure[3], were shown to be

Table 1. Substrate specificity of alkaline phosphatases' (ALP I and ALP II) activity*

Substrate	Relative rate of hydrolysis (%) ALP I	ALP II	Relative activity ALP II/ ALP I
pNPP	100	100	1.50
G-1-P	10.3	15.5	1.50
G-6-P	10.7	15.5	1.40
F-6-P	17.2	24.2	1.40
FDP	24.1	33.3	1.38
GP	13.4	17.5	1.30
ATP	125	157.5	1.26
ADP	101.7	121.2	1.19
AMP	86.2	106	1.22
cAMP	3.4	4.3	1.26
GTP	224.1	280	1.24
GMP	112	151.5	1.35
UTP	137.9	181.8	1.31
UMP	74.1	84.8	1.14
ITP	160.3	203	1.26
IMP	86.2	121.2	1.40
CMP	73.2	106	1.44
XMP	120.6	160.6	1.33
DNA	3.4	3.9	1.14
dnDNA	4.8	6.0	1.25
RNA	4.8	5.6	1.16

*The various compounds except DNA had the same concentration as pNPP (50 µM) and the rate of hydrolysis relative to that of pNPP was determined using the same concentration of enzyme (15 µg) for each reaction mixture. Each value is the mean of 3 separate assay determinations. Abbreviations: paranitrophenol phosphate, pNPP; glucose-1-phosphate, G-1-P; glucose-6-phosphate, G-6-P; fructose-6-phosphate, F-6-P; fructose 1.6 diphosphate, FDP; glycerol phosphate, GP; inosine 5′ triphosphate, ITP; xanthosine 5′ monophosphate, XMP; dnDNA, denatured DNA.

homogeneous on polyacrylamide gel after electrophoresis. In terms of activity ALP II with lower mol. wt seemed more active than ALP I with higher mol. wt when p-NPP was the substrate[3].

Substrate specificity of ALP I and ALP II (table 1). When various phosphorylated compounds were used as substrates for the 2 enzymes (ALP I and ALP II), it was found that the 2 enzymes exhibited properties typical of nonspecific phosphatases. They showed broad substrate specificity. The 2 enzymes had little DNase and RNase activities. The activity of ALP II was greater than that of ALP I with respect to all physiological substrates tested. The activity of ALP II seemed to be higher than that of ALP I by a factor of 1.32 ± 0.18 for these phosphorylated substrates.

Effect of activators on the activities of ALP I and ALP II (table 2). Some compounds, NAD, NADP, DTNB were found to activate ALP I and ALP II when each of these compounds was included in the incubation mixture of each enzyme separately. DTNB seemed to activate the enzymes more than the other compounds. ALP I seemed to be more activated than ALP II when each of these compounds was included in the incubation mixture of each enzyme separately. When NADP replaced pNPP (at the same concentration) as the substrate the enzymes had no activity (approximately 2% of the normal activity with pNPP as substrate).

Effect of thiol reagents on the activities of ALP I and ALP II (table 3). Iodoacetate, mercaptoethanol, dithiothreitol (DTT) inhibited the activities of ALP I and ALP II to various extents. ALP I seemed more sensitive to inhibitions by these thiol reagents than ALP II. $NaBH_4$ had no effect on the activities of ALP I and ALP II.

Discussion. ALP I and ALP II displayed properties typical of nonspecific phosphatases in terms of substrate specificity[8,9]. It is quite interesting to note that the 2 enzymes displayed high activities towards GTP (table 1). Other nucleotides are well utilized with the exception of cAMP, which is poorly metabolized by ALP I and ALP II. The apparent inability of ALP I and ALP II to metabolize cAMP suggests inaccessibility of the cyclic phosphate. High activities of ALP I and ALP II towards nucleotide triphosphates suggest preference for pyrophosphate ester bonds far removed from the sugar moiety.

Hexose phosphates are poorly metabolized. The ability of ALP I and ALP II to utilize nucleotides well is in contrast to the ALP of *Neurospora crassa*[8]. The high relative activities of ALP I and ALP II towards nucleotides may suggest strong regulatory roles in a given physiological state of *A. niger* mycelia. It is still quite likely that ALP II is a derivative form of ALP I but the possibility cannot be ruled out that the enzymes are compartmentalized in the mycelia of *A. niger* in a way similar to that described in earlier reports for *Escherichia coli*[10] and *Salmonella typhimurium*[11]. It is well known that phosphatases can exist as an oligomer of subunits[12] but there is no strong evidence as yet that the ALP I of *A. niger* exists as an oligomer of the smaller ALP II.

The stimulation of activity by the presence of NAD or NADP in incubation mixtures of ALP I or ALP II suggests a link of these enzymes with energy-requiring pathways in the mycelia. It is worthwhile to point out that we have reported earlier that the presence of inorganic phosphate in the growth medium did not repress the production of the phosphatases[3]. The positive effect of DTNB in stimulating activities of ALP I and ALP II suggests the possible involvement of a sulphydryl group in catalytic activity. The slight inhibition shown by the presence of iodoacetate in incubation mixtures of ALP I or ALP II tends to suggest the possible presence of a sulphydryl group involved in catalytic activity. Iodoacetate is well known as a blocking agent of SH groups. DTT and mercaptoethanol inhibit both enzymes, with DTT being more inhibitory on ALP I. DTT and mercaptoethanol are good agents for reducing S-S groups to sulphydryl groups. It is quite likely that such S-S bonds are not required to be reduced for catalytic activity. ALP II was more active than ALP I for all physiological substrates tested (table 1); both enzymes tend to be activated in the presence of DTNB (table 2), and ALP I is more sensitive to inhibitors (table 3). From these observations it seems to us that the idea that these phosphatases (ALP I and ALP II) are scavenging enzymes is not attractive. The near constant factor of the relative activities of the two phosphatases suggests regulatory roles. As at present we have no evidence as to the actual localization of ALP I and ALP II in the mycelia or how they are expressed.

Table 2. Effect of NAD, NADP and DTNB on the activities of ALP I and ALP II

Activator	Relative rate of hydrolysis (%) ALP I	ALP II
None	100	100
NAD	109.7	107.5
NADP	108.5	101
DTNB (dithiobisnitrobenzoic acid)	131.7	117

Each compound was added to the normal incubation mixture as described in the text and the rate of hydrolysis relative to that of pNPP alone (without activator) was determined. The concentration of each activator was the same (20 µM) and the enzyme concentration (15 µg) was the same for each determination. Each value is the mean of 3 separate assay determinations.

Table 3. Effect of thiol reagents on the activities of ALP I and ALP II

Reagent	Relative rate of hydrolysis (%) ALP I	ALP II
None	100	100
Iodoacetate	93.2	98.4
Mercaptoethanol	81.9	92.9
DTT (dithiothreitol)	79.8	92.0
$NaBH_4$ (sodium borohydride)	100	100

Each reagent was added to the normal incubation mixture as described in the text and the rate of hydrolysis relative to that of pNPP alone (without reagent in incubation mixture) was determined. The concentration of each reagent was the same (20 µM), and the enzyme concentration (15 µg) was the same for each determination. Each value is the mean of 3 separate assay determinations.

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