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Penicillium chrysogenum extracellular acid phosphatase: purification and biochemical characterization

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An extracellular acid phosphatase (EC 3.1.3.2) from crude culture filtrate of *Penicillium chrysogenum* was purified to homogeneity using high-performance ion-exchange chromatography and size-exclusion chromatography. SDS-PAGE of the purified enzyme exhibited a single stained band at an M_r of approx. 57000. The mobility of the native enzyme indicated the M_r to be 50000, implying that the active form is a monomer. The isoelectric point of the enzyme was estimated to be 6.2 by isoelectric focusing. Like acid phosphatases from several yeasts and fungi the Penicillium enzyme was a glycoprotein. Removal of carbohydrate resulted in a protein band with an M_r of 50000 as estimated by SDS-PAGE, suggesting that 12% of the mass of the enzyme was carbohydrate. The enzyme was catalytically active at temperatures ranging from 20 °C to 65 °C with a maximum activity at 60 °C and the pH optimum was at 5.5. The Michaelis constant of the enzyme for *p*-nitrophenyl phosphate was 0.11 mM and it was inhibited competitively by inorganic phosphate ($k_i = 0.42$ mM).

Introduction

When yeasts or fungal cells have to utilize organic compounds as phosphate source, this phosphate must be removed from the organic substrate in a reaction catalysed by various phosphatases. Acid phosphatases (EC 3.1.3.2) which catalyse the hydrolysis of a variety of phosphate esters are widely distributed in yeasts or fungi but also in bacteria and tissues [1,2].

A number of studies have been reported on enzymatic characterization and genetic analysis of acid phosphatases from several yeasts and fungi. Acid phosphatases from *Saccharomyces cerevisiae* were found mainly associated with the cell wall [3,4], and two tandemly repeated structural genes (*PHO3* and *PHO5*) have been characterized, while the existence of a third (*P56*) has been suggested on the bases of a 56000 polypeptide [5]. The synthesis of the PHO5 and P56 gene products can be repressed by inorganic phosphate, while the PHO3 gene product is synthesized constitutively [5]. In the fission yeast *Schizosaccharomyces pombe* an acid phosphatase has been identified which was found to be secreted in the growth medium, but unlike to the enzyme from *S. cerevisiae*, the *Sch. pombe* phosphatase has significant activity in the presence of phosphate [6].

Two extracellular acid phosphatases from *Aspergillus ficuum* have been isolated and characterized recently. One has a pH optimum at 2.5 and the other at 6.0 [7,8] and both have been shown to be competitively inhibited by inorganic phosphate [7,8].

A. niger was found to produce three forms of acid phosphatases [9]. The purification and biochemical properties of an intracellular form was described [9] and the gene coding for a secreted form of acid phosphatase, which was phosphate repressible, was isolated [10].

From *P. funiculosum*, a secreted acid phosphatase was described [11], but in contrast to the enzymes mentioned above this acid phosphatase is a phosphohydrolase that acts on both phosphodiesters and phosphomonoesters.

In this paper we report the purification of an extracellular pH 5.5 optimum acid phosphatase from *P.*

Abbreviations: PNGase F, peptide *N*-glycohydrolase; TMFS, trifluoromethanesulfonic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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chrysogenum and data about its molecular and catalytical properties.

Materials and Methods

Materials

p-Nitrophenyl phosphate, *p*-nitrophenyl phenyl phosphonate, bis(*p*-nitrophenyl) phosphate, α-naphtyl acid phosphate, fast garnet GBC salt and standard proteins used in the SDS-polyacrylamide gel electrophoresis and M_r studies were from Sigma (F.R.G.). Nitrocellulose membrane (BA 83; 0.2 μm) was from Schleicher & Schüll, Dassel. Glycan detection kit was from Boehringer, Mannheim. Endoglycosidase H was from USB, Cleveland. Recombinant peptide N-glycosidase F was from Genzyme Corporation, Boston. All of the other reagents were of analytical grade and obtained from Merck, Darmstad (F.R.G.).

Culture

The filamentous fungus *P. chrysogenum*, strain Q176, was grown aerobically at 25° C for 48 h in a medium containing 3 g/l $NaNo_3$, 0.5 g/l $MgSO_4 \times 7H_2O$, 0.1 g/l $FeSo_4 \times 7H_2O$, 20 g/l sucrose in 0.25 mM phosphate, 25 mM citrate buffer (pH 4.8). Cultivations were carried out in 1-l Erlenmeyer flasks (total volume 250 ml) on a rotary shaker (150 rpm).

Purification of acid phosphatase

All purification steps were carried out at 4° C.

Step 1. Ammoniumsulfate precipitation: The mycelia were removed by filtration and discarded. Solid ammonium sulfate was added to the filtrate until saturation, and stirred overnight at 4° C. The precipitate was collected by centrifugation at 40000 × *g* for 30 min. The redissolved precipitate was dialysed overnight against several changes of 50 mM Tris-HCl (pH 8.0) at 4° C.

Step 2. High-performance ion-exchange chromatography: The protein solution was applied to a TSK DEAE-5-PW column (75 × 7.5 mm) equilibrated with 50 mM Tris-HCl (pH 8.0). Proteins were eluted from the column at a flow rate of 1.5 ml min^{-1} by increasing the salt concentration from 0 to 1 M NaCl. Fractions containing acid phosphatase activity were pooled and concentrated by ultrafiltration.

Step 3. High-performance size-exclusion chromatography: The concentrated protein solution was applied to a BIO-Sil TSK 250 column (600 × 2.5 mm) equilibrated with 50 mM Tris-HCl (pH 7.0), 100 mM NaCl. Fractions containing acid phosphatase activity were pooled and stored at −80° C.

Enzyme assays

Spectrophotometrically. The enzyme activity was determined using *p*-nitrophenyl phosphate as substrate. The reaction mixture contained in a total volume of 1 ml: 10 μmol of the substrate, 100 μmol of sodium acetate buffer (pH 5.5), and various amounts of the enzyme. After 5 min of incubation at 30° C, the reaction was stopped by the addition of 2.0 ml of 0.5 M NaOH and the amount of *p*-nitrophenol released was measured at 405 nm [12]. One unit of activity was equal to the amount of enzyme that hydrolyses 1 μmol of *p*-nitrophenyl phosphate per min under standard conditions. A molar absorption coefficient of $18.8 \cdot 10^3$ M^{-1} cm^{-1} for *p*-nitrophenol at 405 nm was taken for calculation of activity.

Zymogram. For detection of enzymatic activity in polyacrylamide gels, preparations of purified enzyme were subjected to electrophoresis under native conditions in gels containing 9% acrylamide using a basic buffer system according to Davis [13]. Phosphatase activity was detected by incubation of the gels in 0.6 M sodium acetate buffer (pH 5.0) containing 4 mM α-naphtyl acid phosphate, 6 mM fast garnet GBC salt for 15 min at room temperature. The reaction was stopped by immersing the gels in 20% methanol, 7.5% acetic acid [14].

Protein determination

Protein was determined according to the method of Bradford [15] using crystalline bovine serum albumin as standard.

Polyacrylamide gel electrophoresis and isoelectric focusing

SDS-PAGE was performed in slab gels containing 10% acrylamide according to Laemmli [16] using the Bio-Rad Mini Protean II gel electrophoresis system. For determination of M_r before and after deglycosylation 8–20% polyacrylamide gradient gels were used [17]. Proteins of the SDS-70 kit from Sigma were used as *M* markers. The isoelectric point (p*I*) of the acid phosphatase was determined with a LKB Multiphor apparatus using broad range pH (3.5–9.5) thin-layer polyacrylamide gels [18]. Appropriate Pharmacia calibration kits were used. Proteins were stained with Coomassie brilliant blue and densitometric scans were used to assess the p*I*.

Glycoprotein staining

Glycoprotein detection was performed using the Glycan Detection Kit from Boehringer, Mannheim. Adjacent hydroxyl groups of glycoconjugates were coupled to digoxigenin after oxidation with periodate as described by the suppliers manual. The modified enzyme was subjected to SDS-PAGE and electroblotted to nitrocellulose as described [19]. The digoxigenin-labelled glycoconjugate was subsequently detected in an enzyme immunoassay using an antibody-alkaline-phosphatase-conjugate.

Deglycosylation

Removal of carbohydrate using recombinant PNGase F was carried out by a modification of the procedure of Elder and Alexander [20]. After denaturation of 3 μg of purified acid phosphatase by boiling in 0.5% SDS, 50 mM β-mercaptoethanol and 100 mM sodium phosphate (pH 8.0) for 5 min the sample was adjusted to 1.25% Nonidet P-40, 16 mM β-mercaptoethanol and 0.16% SDS and incubated overnight with 1 unit of recombinant PNGase F at 37 °C. Digestion was terminated by boiling for 5 min.

For deglycosylation using endoglycosidase H 3 μg of purified acid phosphatase was incubated with $2 \cdot 10^{-3}$ units overnight at 37 °C in 50 mM sodium acetate buffer (pH 5.5). The reaction was stopped by boiling for 5 min [21].

Chemical deglycosylation with anhydrous TFMS was performed as outlined by Edge et al. [22].

Amino acid analysis

Purified acid phosphatase was subjected to acidic hydrolysis and the amino acid analysis was performed as described [23]. Amino acids were detected as their OPA (ortho-phtaldialdehyde/mercaptoethanol) derivates on a Waters HPLC system equipped with a Shimazu RF 530 fluorescence detector. Cysteine residues were determined after performic acid oxidation as described [24]. Tryptophan was determined after hydrolysis in 6 M HCl in 7% (w/v) thioglycolic acid according to Melzer et al. [25] and proline was determined as described by Bohlen and Mellet [26].

Results and Discussion

Acid phosphatase purification

A summary of a typical purification procedure of the extracellular acid phosphatase from *P. chrysogenum* is shown in Table I. A 19-fold purification was achieved by a combination of high-performance ion-exchange chromatography and high-performance size-exclusion chromatography. The overall recovery of the enzyme was about 50%. The progress of purification was monitored by SDS-PAGE (Fig. 1) and the purified enzyme was detected as a single band. A single band was also detected in native-PAGE and activity staining

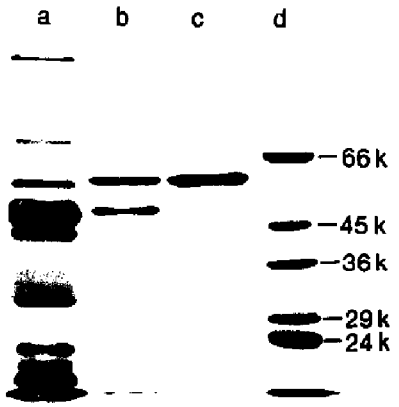

Fig. 1. Electropherogram obtained after SDS-PAGE at different stages of purification: lane a, 15 μg cleared filtrate; lane b, 4 μg of proteins after ion-exchange chromatography; lane c, 4 μg acid phosphatase after purification by gel permeation chromatography; lane d, M_r markers. Proteins were stained with Coomassie brilliant blue.

confirmed that this protein band was the active acid phosphatase (Fig. 2). The fact that only a 19-fold purification was sufficient to achieve homogeneity demonstrates that the acid phosphatase is one of the dominant species of secreted proteins in a *P. chrysogenum* culture filtrate under conditions of limited phosphate growth.

Molecular properties

Molecular weight and isoelectric point. The molecular weight of the native enzyme was estimated by gel permeation chromatography on a Bio-Gel (Bio-Rad) A 1.5 column (8 × 800 mm), calibrated with ferritin (450000), aldolase (160000), bovine albumin (67000) and chymotrypsinogen A (25000). The M_r was estimated to be 50000 ± 5000. Electrophoresis in polyacrylamide gradient gels in the presence of SDS and mercaptoethanol revealed one single band with an M_r of 57000 ± 1000, thus indicating that the native enzyme is a monomer (Fig. 1c).

A monomeric structure of the native enzyme was also reported for the pH 6.0 optimum acid phosphatase of *A. ficuum* [8]. In contrast, acid phophatases from *S.*

TABLE I

Purification of acid phosphatase from P. chrysogenum

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery yield (%)	Purification (fold)
Cleared filtrate	217	11.4	19	100	1.0
Ammonium sulfate precipitation	206	9.8	21	95	1.1
DEAE-ion-exchange HPLC	152	0.5	304	70	16.0
Size-exclusion HPLC	109	0.3	364	50	19.2

Fig. 2. Polyacrylamide gel electrophoresis under native conditions: lanes a and c, cleared filtrate; lanes b and d, purified enzyme; lanes a and b, stained by Coomassie brilliant blue; lanes c and d stained for activity as described in Material and Methods.

cerevisae and pH 2.5 optimum acid phosphatase from *A. ficuum* are known to be active in dimeric form [27,7].

Like most of the acid phosphatases from yeasts and fungi the Penicillium enzyme is a glycoprotein as judged by a positive glycoprotein stain (not shown). Since the different phosphatases contain various amounts of carbohydrate it was of interest to determine the extent of glycosylation of the Penicilium enzyme. This was examined by measuring the shift in apparent M_r of the protein after enzymatical or chemical deglycosylation using endoglycosidase H, recombinant PNGase F and acid TFMS as determind by SDS-PAGE. The three different procedures we used have been shown to remove approx. 90–100% of carbohydrate from various glycoproteins with little or no damage to the polypeptide chain [20].

The effect of endoglycosidase H, recombinant PNGase F and TFMS on the acid phosphatase is shown in Fig. 3. The three methods of deglycosylation fully agreed in their estimate of the polypeptide chain length of acid phosphatase. They resulted in sharp bands on the SDS-PAGE that migrate with an M_r of 50000. Interpretation of the deglycosylation experiments in terms of the weight percentage of carbohydrate content of an enzyme requires accurate estimation of the M_r of the glycosylated protein. Since electrophoresis in single concentration polyacrylamide SDS-gels can give erroneous M_r of glycoproteins we have used gradient gels according to Lambin and Fine [17]. As indicated above, an M_r of 57000 for the glycosylated protein was found using this method. Based on this and the data derived from deglycosylation studies we conclude that the subunit is approx. 12% by weight carbohydrate. The extent of carbohydrate of the Penicillium enzyme is thus less than reported for other secreted acid phosphatases for which up to 50% of glycosylation was found [7,8,27]. However, it should be mentioned that most of the data reported concerning the extent of carbohydrate in fungal or yeast acid phosphatases have not been proved by deglycosylation studies.

The enzymatic digestion with endoglycosidase H and recombinant PNGase F suggests the presence of *N*-linked high mannose-oligosaccharides in the Penicillium acid phosphatase [20].

The isoelectric point of the purified acid phosphatase was estimated to be 6.2 by isoelectric focusing.

Amino acid composition

The results of the amino acid analysis are summarized in Table II. It is obvious that the amino acid composition of the enzyme reveals an unusually high aspartate content. Similar high aspartate contents were also found for acid phosphatases of *S. cerevisiae*, *A. niger* and *A. ficuum* [5,9,8]. On the other hand, we did not find unusually high serine and proline content as reported for the enzymes from *A. ficuum* [7,8].

We have compared the amino acid composition of the Penicillium enzyme with that of *A. ficuum* [7,8], *A. niger* [10], *Sch. pombe* [6] and *S. cervisiae* [5] using the composition divergence according to Haris and Teller [28]. A composition divergence of 49 was found between the Penicillium enzyme and both of the enzymes from *S. cerevisiae*. Values as low as this have been associated with evolutionary homology of proteins as discussed by Blake and Harris [28,29]. The values of composition divergence between the Penicillium enzyme and the other enzymes mentioned above were found to be well above 55. They were therefore too

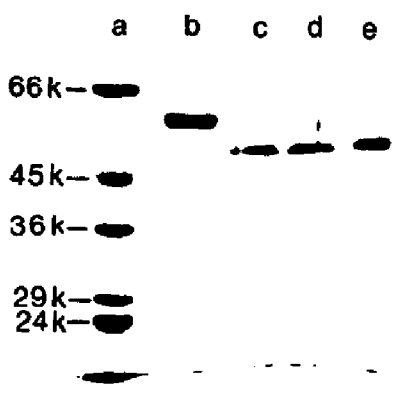

Fig. 3. Effect of deglycosylation on acid phosphatase. Samples were subjected to electrophoresis in SDS polyacrylamide gradient gels as described in Material and Methods. Lane a, M_r markers; lane b, original acid phosphatase glycoprotein; lane c, acid phosphatase after deglycosylation by endoglycosidase H; lane d, after deglycosylation by PNGase F; lane e, after treatment with TFMS.

high to deduce any relationship since there is a lack of correlation between sequence difference and composition divergence above a value of 55 [28,29].

500 pmol of the purified enzyme was used for amino terminal sequence analysis on a microsequenator. Several cycles of the sequenator failed to yield a defined amino acid peak, although an amino acid analysis of the membrane to which the enzyme was bound indicated that enough protein was bound, suggesting that the amino terminus of the enzyme was blocked.

Enzymatic properties

Kinetics. The enzyme was a classic acid phosphomonoesterase, demonstrating no hydrolytic activity with di- or triphosphate esters. Maximal activity was obtained with *p*-nitrophenylphosphate, and the Michaelis-Menten constant for this substrate was 0.11 mM. Inorganic orthophosphate, one of the products of acid phosphatase action, competitively inhibited the enzyme reaction with a k_i of 0.42 mM. In this respect the Penicillium enzyme closely resembled acid phosphatases from *S. cerevisiae*, *A. ficuum*, *A. nidulans* and *P. funiculosum* [27,7,8,30,11].

Optimum pH and effect of temperature on enzymatic activity. The pH activity profile of the Penicillium enzyme is shown in Fig. 4. As can be seen the enzyme showed activity from pH 3.5–6.5, with an optimum at pH 5.5. The enzyme lost its activity rapidly at a pH below 3.5. It had no substantial activity above pH 7.0, which seems to be characteristic for acid phosphomonoesterases.

TABLE II

Amino acid composition of acid phosphatase from P. chrysogenum

The mol%-values are means ± S.D. of three samples. The res/mol-values are based on an M_r of 50000 for the unglycosylated protein.

Amino acid	mol%	Res/mol
Asx	16.2±1.1	74
Glx	7.6±0.4	35
Ser	7.0±0.2	32
His	3.1±0.1	14
Gly	8.3±0.3	38
Thr	7.6±0.4	35
Arg	2.6±0.1	12
Ala	7.9±0.4	36
Tyr	3.9±0.5	18
Cys	2.6±0.1	12
Met	1.1±0.1	5
Val	5.7±0.3	26
Phe	4.6±0.1	21
Ile	3.7±0.1	17
Leu	8.3±0.1	38
Lys	4.4±0.2	20
Pro	3.9±0.2	18
Trp	1.5±0.1	7

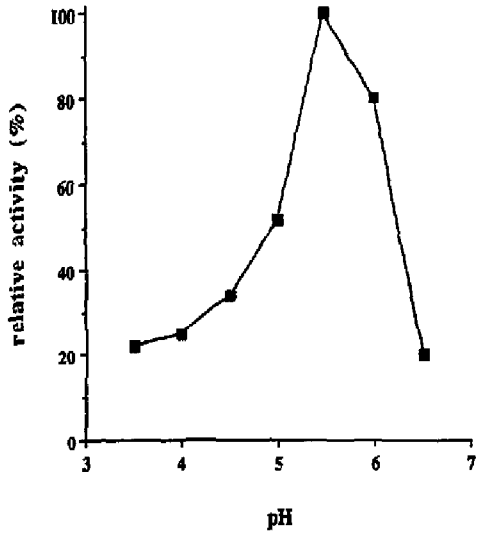

Fig. 4. Effect of pH on the activity of acid phosphatase. Assays were performed in 0.1 M sodium acetate containing 0.3 μg acid phosphatase at 30 °C and the incubation time was 5 min.

The effect of temperature on phosphatase activity is shown in fig. 5. Activity was tested from 10 °C to 70 °C using the standard assay. Maximum activity was observed at 60 °C, which was high above the optimum growth temperature of *P. chrysogenum* at 25 °C. At 10 °C the enzyme activity was 20% and at 30 °C it was 45% of maximum. When analysed at 70 °C the enzyme had lost 90% of its activity. A similar high temperature optimum was reported for the acid phosphatases of *A. ficuum* [7,8], while most of the other phosphatases characterized showed temperature optima of 25–40 °C [9,30].

In order to assess the thermal stability, acid phosphatase was preincubated at 60 °C for a period of 5, 10 and 15 min and cooled to room temperature. The remaining activity was found to be 73, 67 and 43%, respectively. Preincubation at 70 °C for 10 min totally inactivated the enzyme.

Inhibitors

As shown in Table III the Penicillium acid phosphatase did not require any metal ions for activity and was not inhibited by metal chelators. It was inhibited by addition of 10 mM $HgCl_2$ or $ZnCl_2$. L(+)-Tartrate which is a competitive inhibitor of several acid phosphatases from animal sources did not inhibit the Penicillium enzyme [31]. Insensitivity against L(+)-tartrate was also reported for acid phosphatases from *A. ficuum* and *P. funiculosum* [7,8,11]. DTT excerted no effect on the enzyme activity, which seems to be a

TABLE III

Effect of various substances on the activity of the Penicillium phosphatase

Activities are mean ranges of two determinations.

Substance	Final concentration	Activity (% of control)
$CaCl_2$	10 mM	101 ± 2
$MgCl_2$	10 mM	98 ± 1
$MnCl_2$	10 mM	95 ± 3
Sodium tartrate	10 mM	102 ± 4
Phosphomycin	10 mM	75 ± 3
EDTA	10 mM	100 ± 4
DTT	10 mM	97 ± 2
SDS	0.1%	0
$HgCl_2$	10 mM	65 ± 2
$ZnCl_2$	10 mM	31 ± 1

typical feature of plant and microbial acid phosphatases [7,8,11].

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