Substrate analysis and molecular cloning of the extracellular alkaline phosphatase of *Streptomyces griseus*

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*Streptomyces* species secrete large amounts of alkaline phosphatase (AP) enzymes that have not been characterized so far. An AP has been purified to homogeneity from cultures of *Streptomyces griseus* IMRU 3570. The enzyme has a monomer size of 62 kDa and is processed in the culture to a 33 kDa protein as shown by immunoblotting. The enzyme was purified by ammonium sulfate precipitation, CM-Sephadex cationic exchange, chromatofocusing and HPLC Spherosil 3000SW filtration. The pure enzyme uses a variety of organic phosphorylated compounds as substrates. The N-terminal end of the mature protein was found to be RLREDPFTLGVASGDPHP. The gene *phoA* has been cloned using as probe an oligomer based on the N-terminal sequence of the *S. griseus* AP. *phoA* encodes a protein of 62678 Da with low homology to the AP of *Escherichia coli*. The *phoA* gene was found to be homologous to three alkaline-phosphatase-encoding genes previously identified in the *Streptomyces coelicolor* genome. On the basis of the optimal pH, substrate specificity and differences in amino acid sequence of motifs defining the active centre of APs, the *S. griseus* AP uses a wide range of organic phosphate substrates and is different from the phosphatases of Gram-negative bacteria.

**Keywords:** phytases, extracellular enzymes, protein purification, gene cloning

INTRODUCTION

Organic and inorganic phosphates are essential components of living organisms. They are found as orthophosphate, pyrophosphate, polyphosphate, nucleotides, sugar phosphates and phosphorylated derivatives of other organic compounds (e.g. phytic acid). In spite of its relative abundance in nature, phosphate is sometimes a growth-limiting factor for soil micro-organisms because much of its natural supply occurs as insoluble salts. To cope with phosphate limitation, most bacteria cause much of its natural supply occurs as insoluble salts. To cope with phosphate limitation, most bacteria need to acquire phosphate through active transport systems. The GenBank accession number for the sequence reported in this paper is AJ278740.

Inorganic phosphate regulates negatively the biosynthesis of many antibiotics and other secondary metabolites (Liras *et al.*, 1977; Martín *et al.*, 1994). Phosphate regulation of the biosynthesis of candidin (a polyene macrolide antifungal antibiotic) by *Streptomyces griseus* IMRU 3570 has been extensively studied (reviewed by Martín, 1989; Martín *et al.*, 1994). This strain produces high levels of alkaline phosphatase (AP) activity (Daza *et al.*, 1990) in parallel with candidin biosynthesis, under phosphate-starvation conditions. Preliminary evidence indicated that phosphate represses

**Abbreviations:** AP, alkaline phosphatase; PNPP, p-nitrophenyl phosphate; X-phosphate, 5-bromo-4-chloro-3-indolyl phosphate.
transcription of candidcid biosynthesis genes (Asturias et al., 1990) by a mechanism similar to that of phosphate repression of AP. Some mutants of S. griseus 3570 deregulated in phosphate control of candidcid biosynthesis (Martin et al., 1979) are also derepressed in AP.

Characterization of the S. griseus AP was important as the first step to clone the phoA gene by reverse genetics. Availability of the phoA gene will allow us to study the phosphate regulation of this extracellular enzyme at the transcriptional level in order to compare it with the existing knowledge on phosphate regulation of secondary metabolism in this strain (Asturias et al., 1990).

In this work we describe the purification to homogeneity and substrate kinetics of the extracellular S. griseus AP and the cloning and analysis of the phoA gene.

METHODS

Micro-organisms and culture conditions. S. griseus IMRU 3570, the candidcid producer (Liras et al., 1977), was used as the source of AP. To grow this strain, 10⁶ spores were inoculated in 250 ml YED medium (containing, per litre: yeast extract 10 g, glucose 10 g; pH 7.0) for 36 h, and 5 ml of this culture was used to seed 100 ml SPG medium (containing, per litre: soybean meal 25 g, glucose 60 g, ZnSO₄·7H₂O 0.143 g; pH 7.5) in 500 ml baffled flasks. After 48 h growth in an orbital shaker (Gallenkamp) at 32 °C and 220 r.p.m., the mycelium was removed by centrifugation at 8500 g for 30 min and the supernatant was used for the purification of the enzyme.

To study production of AP in defined conditions, asparagine-minimal medium was used (asparagine 25 g, d-glucose 5.5 g, MgSO₄·7H₂O 0.123 g, FeSO₄·7H₂O 1.39 μg, ZnSO₄·7H₂O 1.43 μg, distilled water 1000 ml; pH 7.6) (Martin & McDaniel, 1975).

Alkaline phosphatase assay and phosphate determination. AP activity (EC 3.1.3.1) was measured as follows. Ten microlitres of enzyme solution was added to 50 μl 25 mM Tris/HCl buffer pH 9.5 containing 10 mM p-nitrophenyl phosphate (PNPP) and 0.4 mM CaCl₂, and incubated at 30 °C for different times. The reaction was stopped by adding 2 ml 0.5 M Na₂CO₃ and the absorbance of the p-nitrophenol formed was measured at 410 nm. One unit of enzyme is defined as the activity that forms 1 μmol p-nitrophenol min⁻¹. The specific activity is given as units (mg protein)⁻¹. Phosphate was determined by the Fiske–SubbaRow method (Leloir & Cardini, 1957) using sodium phosphate buffer as standard. Protein concentrations were measured with the Bradford reagent (Bio-Rad).

Hydrolysis of different substrates. The phosphatase-mediated release of inorganic phosphate from different substrates was determined by incubating each substrate at 10 mM concentration for 10 min at 30 °C with pure AP; the reaction was stopped by boiling the samples. Inorganic phosphate released by the phosphatase was quantified by the Fiske–SubbaRow method as the difference in phosphate present in the samples at zero time and after 10 min reaction.

Chemicals. Phenylmethylsulfonyl fluoride (PMSF), and the substrates PNPP (disodium hexahydrate), phytic acid (myo-inositol hexaphosphate), umbelliferone phosphate, α-naphthyl phosphate, β-glycerol phosphate, 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate), glucose 6-phosphate, fluorescein phosphate, tripolyphosphate, ATP, UMP and cAMP used to test enzyme specificity were obtained from Sigma.

Purification of the alkaline phosphatase. SPG culture supernatants (1000 ml) were concentrated in a dialysis bag by treatment with PEG 20000 for 24 h at 4 °C to a final volume of 430 ml without appreciable loss of activity. Ammonium sulfate solution was added to the concentrated enzyme preparation and the activity precipitated in the range of 40–70% saturation. The protein pellet was collected by centrifugation and resuspended in 17.5 ml 25 mM Tris/HCl buffer pH 9.5 containing 0.4 mM CaCl₂, applied to a Sepharcl S-300 column (980 × 26 mm) equilibrated with 100 mM Tris/HCl buffer pH 9.0 and eluted with the same buffer. The active fractions of the eluate were concentrated through P-30 membranes in an Amicon ultrafiltration apparatus. This partially insoluble preparation was then centrifuged at 10000 g for 10 min. The active protein pellet was solubilized in 3% Triton X-100 and mixed with the previous supernatant. The enzyme preparation was adjusted to pH 7.5 and applied to a cation-exchange CM-Sephadex column (160 × 15 mm) equilibrated with 10 mM Tris/HCl buffer pH 7.5. The phosphatase was eluted with a 0–1 M linear NaCl gradient. The active fractions were pooled, and concentrated by filtration through Amicon P-30 membranes; the pH was adjusted to 9.4 with 25 mM ethanolamine pH 9.4 and the pooled eluate applied to a PBE94 (Pharmacia) chromatofocusing column (200 × 20 mm) equilibrated with 25 mM ethanolamine pH 9.4. A pH gradient of 200 ml polybuffer 96/distilled water (1:10, v/v) adjusted to pH 4.0 with acetic acid eluted most of the proteins of the preparation except the phosphatase, which was eluted with 1 M NaCl at pH 4.0.

SDS-PAGE. Denaturing SDS-PAGE was performed as described by Laemmli (1970). Non-denaturing PAGE was carried out in the same system but omitting SDS, β-mercaptoethanol and the boiling treatment of the samples.

Determination of N-terminal amino acid sequence. The phosphatase protein obtained after chromatofocusing was purified to homogeneity by filtration through Amicon P-30 membranes; the pH was adjusted to 9.4 with 25 mM ethanolamine pH 9.4 and the pooled eluate applied to a PBE94 (Pharmacia) chromatofocusing column (200 × 20 mm) equilibrated with 25 mM ethanolamine pH 9.4. A pH gradient of 200 ml polybuffer 96/distilled water (1:10, v/v) adjusted to pH 4.0 with acetic acid eluted most of the proteins of the preparation except the phosphatase, which was eluted with 1 M NaCl at pH 4.0.

Antibodies and immunoblotting. Rabbit antisera were obtained against two different AP preparations. Native enzyme purified through a Sphaerogel TSK 3000SW column (250 μg) in Freund’s complete adjuvant was injected subcutaneously into female New Zealand White rabbits. Three subsequent injections of 125 μg of the protein were administered every 2 weeks in Freund’s incomplete adjuvant. Alternatively, AP (about 200 μg protein) denatured by boiling for 3 min in the presence of 2% SDS and 0.1% β-mercaptoethanol was resolved by SDS-PAGE, the 62 kDa band was cut, equilibrated for 30 min in distilled water, homogenized in saline solution, mixed with Freund’s complete adjuvant and injected into the rabbits. Additional inoculations of 100 μg of protein in Freund’s incomplete adjuvant were administered every 2 weeks. The behaviour of both anti-phosphatase sera in immunoblotting experiments was identical.

Immunoblottings were performed as described by Towbin et al. (1992). The blotted protein was detected with the antiserum using a double-antibody enzyme-conjugate immunodetection method and the colour was developed with nitro blue tetrazolium and X-phosphate.

DNA manipulations. Escherichia coli DH5α was used as host.
The 62 and 33 kDa forms of the extracellular alkaline phosphatase are repressed by phosphate

S. griseus IMRU 3570 showed a very high AP activity when grown in SPG medium for up to 75 h. More than 99% of the phosphatase activity was found in the culture supernatant and peaked at 24–48 h (630 units per mg cell dry weight). Since the complex SPG medium contains solid particles, we studied the production of AP in YED, YEME and asparagine-minimal medium. In all these media the specific AP activity was in the range of 5–10% of that in SPG medium. AP activity severely decreased (97%) when inorganic phosphate (10 mM) was added to SPG medium; by contrast the cell dry weight had increased by fivefold at 48 h after phosphate addition, confirming that the SPG medium is limited by phosphate. Removal of phosphate from the culture supernatant by overnight dialysis did not result in increased enzyme activity, suggesting that phosphate inhibits phosphatase formation.

To confirm inhibition of phosphatase formation, supernatants of 24, 36 and 40 h cultures of S. griseus grown in SPG medium and in SPG supplemented with 10 mM phosphate were analysed by SDS-PAGE (Fig. 1a) and immunoblotted with anti-phosphatase antibodies. Two immunoreacting bands were found in the control cultures (Fig. 1b). One of them corresponds to a protein of 62 kDa (in agreement with the molecular mass determined for the AP monomer: see below) which was found in broth from cultures grown in SPG medium but not in phosphate-supplemented cultures (Fig. 1b, lanes 3 and 4). A second immunoreactive band of 33 kDa was also inhibited by phosphate; it is probably a degradation product of the 62 kDa AP monomer since the 33 kDa form appears with increasing intensity during the fermentation, coinciding with the loss of the 62 kDa band (Fig. 1c).

The extracellular phosphatase forms a high-molecular-mass complex

To determine the molecular mass of the native AP, the preparation obtained by ammonium sulfate precipitation (40–70% saturation) was size-fractioned by chromatofocusing.

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**Table 1. Steps for the purification of the alkaline phosphatase of S. griseus IMRU 3570**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol. (ml)</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Specific activity ([\text{units (mg protein)}^{-1}])</th>
<th>Purification (-fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>1000</td>
<td>894</td>
<td>2620</td>
<td>2.93</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>PEG concentration</td>
<td>430</td>
<td>867</td>
<td>2987</td>
<td>3.44</td>
<td>1.17</td>
<td>114</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation (40–70%)</td>
<td>17-2</td>
<td>319</td>
<td>1592</td>
<td>5.00</td>
<td>1.70</td>
<td>61</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>178</td>
<td>156</td>
<td>831</td>
<td>5.32</td>
<td>1.81</td>
<td>32</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>27</td>
<td>60</td>
<td>447</td>
<td>7.45</td>
<td>2.54</td>
<td>17</td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td>19-7</td>
<td>20</td>
<td>312</td>
<td>15.60</td>
<td>5.32</td>
<td>12</td>
</tr>
</tbody>
</table>
Active samples from the void volume of gel filtration associated with lipids in the supernatant of the culture. Either a multimeric protein or that it is partially first fractions of eluate, suggesting that the enzyme is the void volume and part eluted as a broad peak in the inclusion limit 2000 kDa, part of the activity remained in the preparation was filtered through Sephacryl S-500 (exclusion limit 400 kDa), in which the AP eluted with the void fraction of eluate, giving an enzyme preparation with a single peak with 1 M NaCl solution (Fig. 2a), giving an enzyme preparation of a specific activity 156 units (mg protein)$^{-1}$ (Table 1) and a final recovery of 12%.

The enzyme preparation obtained after HPLC filtration was assayed after non-denaturing 10% PAGE by soaking the gel in buffer containing PNPP. A protein with AP activity unable to penetrate the 10% acrylamide-bisacrylamide was observed in the boundary with the stacking gel. This band was excised from the gel, homogenized in distilled water and applied to: (i) a denaturing SDS-PAGE gel and (ii) a 7-5% non-denaturing gel. A single band of 62 kDa was found in the SDS-PAGE gel after Coomassie blue staining whereas in the native gel AP activity migrated slowly in the gel close to the 440 kDa marker.

**Optimal parameters for alkaline phosphatase activity**

Using a pure phosphatase preparation [36 units (mg protein)$^{-1}$] the reaction on PNPP as substrate was linear for at least 90 min. The activity was not strictly dependent on CaCl$_2$ addition but increased when Ca$^{2+}$ was added to the assay, to a maximum value of 234% in the presence of 0.4 mM CaCl$_2$ with respect to the enzyme preparation without CaCl$_2$ added. This result indicates that the S. griseus AP might be functionally

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**Fig. 2.** (a) Elution of the AP after purification by chromatofocusing in PBE94 gel. ○, $A_{280}$; ●, AP activity; ▲, pH. (b) Purification by preparative HPLC in Sphaerogel TSK3000SW. Absorption at 280 nm (—); AP activity (●). (c) SDS-PAGE of different steps during the purification. Lanes: 1, SPG culture supernatant; 2, supernatant after PEG concentration; 3, precipitate in ammonium sulfate 40–70% saturation; 4, sephacryl S-300; 5, CM-Sephadex; 6, chromatofocusing; 7, active fractions after HPLC Sphaerogel filtration; 8, molecular mass standards (Sigma), sizes in kDa are indicated on the right.
similar to the calcium-requiring Bacillus phytases (Kerovuo et al., 1998).

The optimal pH for the enzyme on PNPP was 9.5, with a sharp decrease in the range of 7.5–9.5 and 11.0–12.5. At pH 11 the activity was less than 85%. The activity was nil at pH 7.0.

The enzyme activity increased with temperature to a maximum at 50 °C. At 1 mM concentration, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$ and Sn$^{2+}$ ions inhibited the activity 95–100% and Mn$^{2+}$ and Co$^{2+}$ exerted a 75% inhibition, but Fe$^{2+}$ and Fe$^{3+}$ were not inhibitory. EDTA produced 100% inhibition, probably by chelating Ca$^{2+}$ ions. Arsenate ions (1 mM) did not affect the phosphatase activity. PMSF at 1 mM and 5 mM caused 40% and 85% inhibition, respectively.

**Substrate specificity**

The S. griseus AP showed a $K_m$ for PNPP of 130 μM. The activity on PNPP was competitively inhibited by inorganic phosphate ($K_i$ 220 μM) and by triply-phosphate. If the activity on PNPP is taken as 100%, the activities on sodium pyrophosphate and pyrophoric acid, under the same assay conditions, were 120 and 187% (however these substrates release two and three phosphate groups respectively). Umbelliferyl phosphate, x-naphthyl phosphate, ATP, UMP and β-glycerol phosphate were used with 54, 39, 23 and 19% efficiency as compared to PNPP. Activities in the range of 7–10% were found with fluorescein phosphate, glucose 6-phosphate, triphosphate, cAMP and X-phosphate as substrates. These results indicate that the S. griseus AP uses a wide range of inorganic and organic phosphorylated substrates.

**The N-terminal sequence of the purified protein shows similarity to alkaline phosphatases**

The semipreparative HPLC Sphaerigel TSK 3000SW filtration step was important to obtain homogeneous AP to sequence the N-terminal end of the protein since it removed two minor contaminating proteins (Fig. 2b) barely visible in Fig. 2(c), lane 6. Two separate sequencing experiments in different laboratories gave a clear sequence of 18 amino acids: RLREDPFTLGVASGDHPH with a single ambiguity in the aspartic acid at position 15. This amino acid sequence was found to be 61% similar to the N-terminal sequence of the PhoD phosphatase of Bacillus subtilis (Eder et al., 1996).

**Cloning of the phoA gene**

SmaI-, SalI-, SacI- or PvuII-digested DNA from S. griseus was transferred to nylon membrane and probed with a 33 nucleotide degenerate oligomer based on the N-terminal amino acid sequence of the APs from S. griseus and B. subtilis. Several strong bands of hybridization were detected. SalI-digested DNA was extracted from the agarose gel in the region giving strong hybridization and used to construct a mini-library in the E. coli vector pUC19. When this plasmid library was probed again with the 33-mer probe, one clone gave a strong hybridization signal and was found to contain a 1.2 kb SalI DNA insert. The nucleotide sequence of the fragment showed that it contained a truncated ORF1 encoding a protein with high similarity to the phosphodiesterase/AP encoded by phoD of B. subtilis and to the sequence of the unpublished AP of Streptomyces tendae (Q9RCK5). To clone the entire ORF1 a 450 bp NotI–Eco72I DNA fragment internal to the cloned ORF was used to screen SmaI-, NotI-, Ncol- or KpnI-digested S. griseus DNA. A 2.3 kb NotI DNA fragment, giving positive hybridization, was found to overlap partially with the 1.2 SalI fragment. This fragment was extracted from the gel and subcloned in pBSKS(+). Four transformants were found by hybridization to contain the 2.3 kb NotI DNA insert. This insert was isolated and completely sequenced.

The complete ORF1 contains 1695 nucleotides and showed a clear preference for codons containing C or G in the third position. It was preceded by a putative ribosome-binding site GAGGAG complementary to the 3′ end of Streptomyces lividans 16S rRNA, located 8–14 nt upstream of the GTG start codon. The ORF encodes a 565 amino acid protein with a deduced molecular mass of 62678 Da. When compared with the proteins of the SWALL database the deduced protein showed a high similarity with the putative AP of S. tendae (72.7% identical amino acids), with the APs of Streptomyces coelicolor (71% identity with Q9RKP2, 65% with Q9XAK7 and 37% with CAB51460), and with phoD of B. subtilis (40.5% identity) (Fig. 3a) throughout the entire sequence of the protein. Therefore, we have named the S. griseus cloned gene phoA and propose the designations phoA, phoB and phoC for the homologous genes of S. coelicolor (Q9RKP2, Q9XAK7 and CAB51460, respectively), following the order of decreasing identity. A fourth ORF (PhoD) encoding a putative phosphatase present in the S. coelicolor genome (Q9XAN2) shows much lower identity (27%).

Surprisingly, there is no clear AP gene in the genome of Mycobacterium tuberculosis (Cole et al., 1998) or in the known sequences of Mycobacterium leprae or Mycobacterium bovis. The highest similarity of any protein in M. tuberculosis with PhoA of S. griseus is only 14% of identical residues. Similarly, no AP homologous to phoA was found in the genome of Corynebacterium diphtheriae (FASTA3, SWISS-PROT database).

**N-terminal region, active site and motifs of interest in PhoA of S. griseus**

The amino acid sequence determined from the N-terminal end of the pure protein corresponds to amino acids 75–90 of the protein encoded by phoA. This region is conserved, especially amino acids 80–90, in the phosphatases of S. tendae, B. subtilis PhoD, and PhoA, PhoB and PhoC from S. coelicolor (motif A in Fig. 3a) but it is absent from the PhoD phosphatase of S. coelicolor.
Fig. 3. (a) Comparison of the amino acids sequence of the PhoA protein of *S. griseus* with PhoA of *S. coelicolor* and PhoD of *B. subtilis*. Lines over the sequence indicate β-sheets A, C, D, G, H, I and the phosphorylation site (S 147) of the *E. coli* and *B. subtilis* enzymes (Hullet et al. 1991). The arrow indicates the cleavage site deduced from the N-terminal end of the mature *S. griseus* AP. (b) Motifs in the active centre of different types of APs: *E. coli* PhoA, *B. subtilis* PhoA, PhoB and PhoD, *S. griseus* PhoA, *S. tendae* PhoA and *S. coelicolor* PhoA and PhoB. Note the differences in amino acid sequence of the AP of *E. coli* PhoA and *Bacillus subtilis* PhoA and PhoB in relation to other phosphatases. In both (a) and (b), conserved residues are in reverse type and asterisks show the amino acids involved in phosphorylation or metal binding according to Hulett et al. (1991).

Amino acid sequences located in the β-sheets A, C, D, F, G, H and I, corresponding to the ligands for metals and the phosphorylation site in the enzyme (Hulett et al., 1991), occur in the APs PhoA and PhoB of *B. subtilis* but interestingly they are not well conserved in the PhoD protein of *B. subtilis* that is the more closely related to the *S. griseus* AP.

Comparison of the *S. griseus* amino acid sequence with those of *E. coli* AP, *B. subtilis* PhoA and PhoB and other putative *Streptomyces* phosphatases suggests that the active centre of the *S. griseus* AP corresponds to amino acids 144–167 with S147 (Fig. 3b) as phosphate-binding site. Serine147 is replaced by threonine in the AP of *S. tendae* and in *S. coelicolor* PhoA.

**DISCUSSION**

Phosphatases are important enzymes for organic phosphate utilization by actinomycetes in soil. In *E. coli* and other Gram-negative bacteria AP is a periplasmic enzyme encoded by *phoA* (Kantrowitz, 1994). In *Streptomyces* species AP is an extracellular enzyme (Daza et al., 1990) that is secreted in very large amounts under phosphate-starvation conditions. Two forms of AP were observed in *S. griseus* cultures by immunoblotting with antibodies against AP. The 62 kDa active form of the AP is converted into a form of about 33 kDa after 24 h growth in SPG medium. Similar processing of extracellular enzyme has been reported for *Streptomyces* α-amylases (García-González et al., 1991), xylanases (Blanco et al., 1997), glucanases or cellulases (Fernández-Abalos et al., 1992).

Production of the *S. griseus* AP protein is strongly decreased by inorganic phosphate, as shown by immunoblotting of the AP in phosphate-limited and phosphate-supplemented cultures. Both forms of the AP were absent from phosphate-supplemented cultures, suggesting that *de novo* synthesis of the AP is inhibited by phosphate. Promoter analysis and transcriptional
studies of the cloned gene are now in progress and will allow us to study the molecular mechanisms of phosphate control of gene expression in *Streptomyces*.

As described in this article the *S. griseus* AP uses a wide range of substrates including inorganic oligophosphates and polyphosphates as well as a variety of organic phosphates. The enzyme is stimulated by Ca$^{2+}$ ions as are the *Bacillus* phytases (Kerovuo et al., 1998; Powar & Jagannathan, 1982) but they lack the RHGE/DRXP motif characteristic of phytases and acid phosphatases (Piddington et al., 1993). Phytases (EC 3.1.3.8) are members of the histidine acid phosphatases subfamily that form phosphorylated protein intermediates during the substrate hydrolysis (Mitchell et al., 1997). Comparison of the *S. griseus* PhoA amino acid sequence with those of other phosphatases revealed very little homology (about 10%) to fungal phosphatases. In *E. coli* there is a phosphatase, encoded by the *appA* gene, that shows acid phosphatase and phytase activity (Golovan et al., 2000), and *B. subtilis* possesses a phosphatase with optimal pH for phytic acid hydrolysis of 7.5 (Powar et al., 1982). However, the optimal pH for the *S. griseus* phosphatase is 9.5 and there are major differences in structure between the *S. griseus* AP and other phytases.

When the *S. griseus* PhoA protein was compared with other proteins in the databases a higher similarity was found with the phosphatase of *S. tendae* and with proteins deduced from the *S. coelicolor* genome sequence. The similarity to the *E. coli* AP was surprisingly low (14% identical residues). Indeed the *E. coli* enzyme is a homodimer that contains two Zn$^{2+}$ ions and one Mg$^{2+}$ ion in each active centre (Kantrowitz, 1994), whereas the *S. griseus* AP was stimulated by Ca$^{2+}$ but was inhibited by Zn$^{2+}$. The *S. griseus* PhoA is more similar to the *B. subtilis* Ca$^{2+}$-dependent phosphatase (Kerovuo et al., 1998; Powar & Jagannathan, 1982), to the phosphatase of *Bacillus amylooligofaciens* that has been crystallized with Ca$^{2+}$ (Ha et al., 1999, 2000) and to the thermostable phosphatase of *Thermus caldophilus* (Park et al., 1999) that is also inhibited by Zn$^{2+}$.

The N-terminal sequence of the mature AP corresponds to amino acids GLRLREDPFTLGVASGDPHP$^{55}$. This N-terminal region is well conserved in the PhoD protein of *B. subtilis*, the PhoA, PhoB and PhoC proteins of *S. coelicolor* and the APs of *S. tendae* but not in the PhoD of *S. coelicolor*. The processing site of the *S. griseus* AP does not correspond to the canonical AXA proposed by von Heijne (1986). However, there are two conserved sequences $^{56}AAAG^{58}$ and $^{63}AGA^{65}$ that may be cleavage sites for signal peptidases and the N-terminal end of the mature AP may originate from additional processing of a pro-AP as has been described for the phytase of *Aspergillus niger* (van Hartingsveldt et al., 1993).

AP signature sequences are conserved in the *S. griseus* AP. The $\beta$-sheet A of the *S. griseus* AP corresponds to amino acids 81–100 (Fig. 3b), where the conserved D$^{88}$ is involved in metal binding in the *E. coli* enzyme. The sequence of the $\beta$-sheet G for several APs is shown in Fig. 3(b). The E$^{388}$ and D$^{392}$ (asparagine in *S. coelicolor* PhoA and *B. subtilis* PhoD) are metal-binding sites in the *E. coli* enzyme and are conserved in *S. griseus* AP. Additional $\beta$-sheets as well as the T$^{206}$ in sheet C, H$^{440}$ in $\beta$-sheet H and H$^{569}$ in $\beta$-sheet I (which does not exist in *E. coli*) have been described as metal-binding sites in *B. subtilis* and *E. coli* APs.

No genes encoding phosphatases with similarity to the *E. coli* AP have been found in the genome of *S. coelicolor*. All this evidence indicates that the extracellular APs of *S. griseus* and other *Streptomyces* species belong to a family of APs only distantly related to the *E. coli* enzyme (Lim et al., 2000). No APs similar to the *S. griseus* AP have been found in the *M. tuberculosis* genome or the available *M. leprae*, *M. bovis* or *C. diphtheriae* sequences, suggesting that the PhoA-encoded AP is specific for soil-living actinomycetes.

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