

Purification and characterization of a hygromycin B phosphotransferase from *Streptomyces hygroscopicus*

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A hygromycin B phosphotransferase activity from *Streptomyces hygroscopicus* has been highly purified by ammonium sulphate fractionation followed by affinity column chromatography through Sepharose-6B–hygromycin-B. The combined active fractions showed a single protein band (41 kDa) when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. When gel electrophoresis was performed under non-denaturing conditions, the single protein band promoted *in situ* phosphorylation of hygromycin B, indicating that this protein corresponded to the purified hygromycin B phosphotransferase. The enzyme has been purified 236-fold and approximate K_m values of 0.56 μM and 36.4 μM for hygromycin B and ATP, respectively, were deduced.

Aminocyclitol antibiotics are strong inhibitors of protein synthesis produced by several species of the actinomycetes [1, 2]. Several of these organisms are self-protected against their toxic products by two types of enzyme-catalysed transferase reactions that inactivate the antibiotics by either phosphorylation or acetylation [2, 3]. *N*-Acetyltransferases modify aminocyclitols at the 6'- and 2'-amino positions of aminoheptose I and the 3-amino position of deoxystreptamine. The antibiotic can also be inactivated by *O*-phosphorylation at the 3'-hydroxyl group of aminoheptose I, the 3'-hydroxyl group of aminoheptose III and at the 6-hydroxyl group of streptidine and 2-deoxystreptidine [1, 2, 4].

Hygromycin B is an unusual aminocyclitol antibiotic in that it is active against both prokaryotic and eukaryotic cells and specifically inhibits protein synthesis at the translocation step [5, 6]. It is produced by *Streptomyces hygroscopicus* which possesses a unique hygromycin B *O*-phosphotransferase (HPH) that fully inactivates the antibiotic by phosphorylation of the 7'-hydroxyl group of the destomic acid moiety [7, 8] thus being a new type of aminocyclitol-*O*-phosphotransferase. *S. hygroscopicus* apparently requires only the HPH activity to provide resistance to hygromycin B [8].

Previous attempts to purify HPH enzyme by conventional column chromatography only resulted in a partially purified, and very unstable, preparation [8]. Similar findings are also common for other aminocyclitol modifying enzymes. However, the introduction of affinity column chromatography techniques allowed an apparently total purification of aminocyclitol phospho and acetyl transferases [9, 10].

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Abbreviations. HPH, hygromycin B phosphotransferase; *hyg*, hygromycin B phosphotransferase gene; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

Enzyme. Hygromycin B phosphotransferase (EC 2.7.1.--).

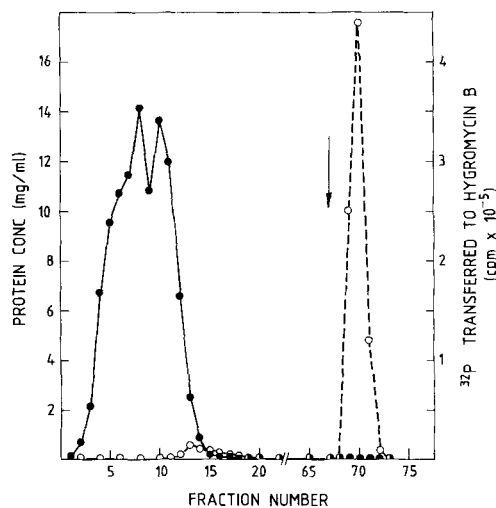


Fig. 1. Sepharose-6B–hygromycin-B affinity chromatography. Chromatography was performed as described in Materials and Methods. The arrow indicates the initial point of addition of hygromycin B. (●) Protein content and (○) HPH activity of the eluted fractions

In this work we report the purification of HPH from *S. hygroscopicus* by affinity column chromatography and its biochemical characterization.

MATERIALS AND METHODS

Purification of hygromycin B phosphotransferase

Streptomyces hygroscopicus NRRL 2387 was grown at 30°C in liquid YEME [11] supplemented with 34% (w/v) sucrose. To prepare cell extracts, 20 g of frozen cells were broken with alumina, resuspended in buffer E [20 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, 400 mM (NH₄)₂SO₄, 1 mM dithiothreitol] and centrifuged for 2 h at 100 000 × g. The

Table 1. Purification of HPH enzyme

Enzyme activity was measured at 10 μM hygromycin B and 300 μM ATP as the amount of ^{32}P transferred to hygromycin B min^{-1} . To remove contaminating hygromycin B in the last stage, the combined active fractions were treated as described in Materials and Methods

Stage of purification	Protein	Total activity	Specific activity	Purification factor
	mg	nmol min^{-1} (%)	$\text{nmol min}^{-1} \text{mg protein}^{-1}$	
100000 \times g supernatant	139.52	295.8 (100)	2.12	1
0–30% $(\text{NH}_4)_2\text{SO}_4$ fraction	122.88	339.1 (114)	2.76	1.3
Sepharose-6B–hygromycin-B	0.064	32.0 (10.8)	500.00	235.8

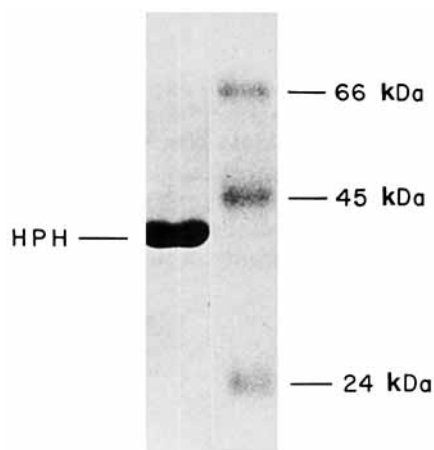


Fig. 2. PAGE of purified HPH enzyme. An aliquot (15 μg protein) of the combined active fractions from the Sepharose-6B–hygromycin-B column (Fig. 1) was subjected to PAGE and the gel stained with Coomassie blue

resulting supernatant was used as a source of HPH enzyme.

Hygromycin B was coupled to epoxy-activated Sepharose 6B according to the supplier's instructions [11a], selecting conditions which favored the reaction of the amino groups of the antibiotic with the Sepharose 6B. The resin (12.5 g) was swollen in distilled water and then resuspended in 150 ml of 90 mM NaOH, pH 12.2, containing 20 mg/ml hygromycin B. The resin was finally equilibrated with buffer E and poured into a column (1 \times 6 cm; 10 ml). This column has been maintained for over a year in the cold room with no loss of activity.

To purify the HPH enzyme, the 100000 \times g supernatant was treated with $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation. The precipitated material was removed by centrifugation and the supernatant (24 ml) was applied to the Sepharose-6B–hygromycin-B column after which the column was washed with 200 ml of buffer E, until the A_{280} of the eluent was zero. The HPH was eluted from the column with a solution containing 2 mg/ml hygromycin B in buffer E. Since the active column fractions contained high concentrations of hygromycin B, they were extensively dialysed at 4°C versus buffer E and then incubated for 30 min at 30°C in the presence of 300 μM ATP. This last treatment was important to remove any remaining traces of hygromycin B. The mixture was dialysed against buffer E to remove unreacted ATP and phosphorylated hygromycin B.

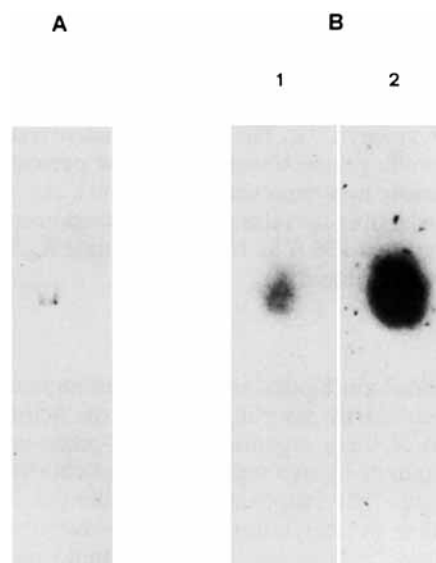


Fig. 3. HPH activity of protein in polyacrylamide gels. HPH, purified by affinity chromatography (two samples), was subjected to gel electrophoresis in a 7.5% polyacrylamide gel under non-denaturing conditions. Half of the gel was used to detect the protein band (A). The other half was used to determine HPH activity (B) as described in Materials and Methods. The phosphocellulose paper was exposed for 6 h (1) or 24 h (2)

Analyses

Protein was estimated as described by Bradford [12].

HPH activity was determined by the radiochemical assay described elsewhere [4, 8]. Blanks were run in the absence of hygromycin B. To estimate reaction velocities, hygromycin B was present at 10 μM and aliquots were taken at time intervals. One unit of HPH activity was defined as the amount of protein catalysing the phosphorylation of 1 nmol of hygromycin B/min at 30°C.

The K_m values of HPH for hygromycin B and ATP were determined using initial velocities. The specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was 720 Ci/mol and the substrate concentrations varied in the range 1–4 μM for hygromycin B and 37.5–300 μM for $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Reaction mixtures (200 μl) were pre-warmed at 30°C and the assays were started by adding 20 mU of purified HPH enzyme. Aliquots of 45 μl were then removed at specified time intervals (0–12 min) to determine phosphorylated hygromycin B.

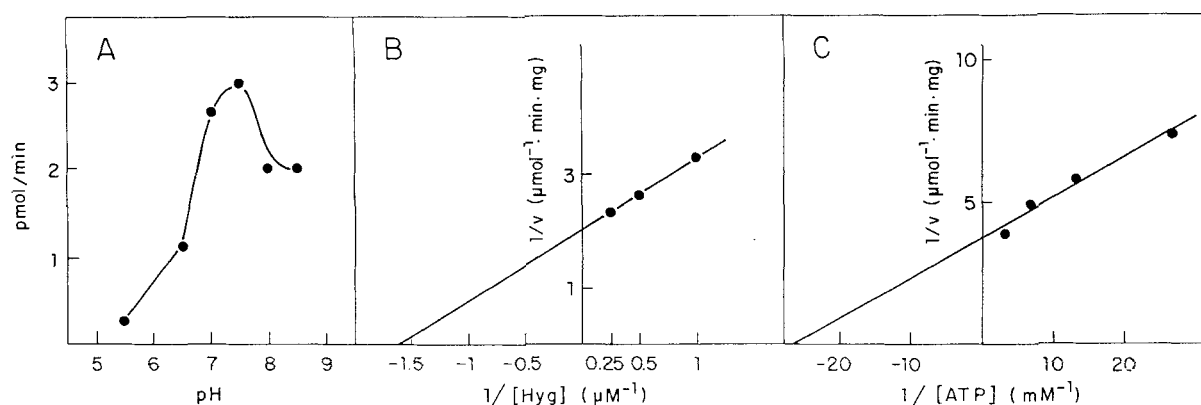


Fig. 4. The effect of pH on HPH activity and kinetic parameters of the HPH enzyme. To estimate the effect of pH (A), the HPH activity was assayed as indicated in Materials and Methods. Buffers used were either 50 mM Mes/NaOH (pH 5.5–6.5) or 50 mM Tris/HCl (pH 7.0–9.0). The K_m for hygromycin B (B) and ATP (C) were estimated by double-reciprocal plots fitted to appropriate rate equations by the least-squares method

Polyacrylamide gel electrophoresis

12% SDS-PAGE was performed as described by Laemmli [13]. Proteins in the gels were stained as described previously [14].

To detect HPH activity in polyacrylamide gels, the enzyme samples were run in PAGE (7.5% polyacrylamide) as described by Laemmli [13] except that SDS was omitted. The borders of the gel were sealed with 1% agarose and covered with a thin layer (0.3 ml/cm²) of 0.7% agarose containing 75 mM Tris/HCl, pH 7.4, 10 mM MgSO₄, 100 mM (NH₄)₂SO₄, 1 mM hygromycin B, 20 mM ATP and 50 μCi [γ -³²P]ATP (3000 Ci/mmol). The gel was maintained for 2 h at 30°C to allow the reaction to proceed. Phosphorylated hygromycin B was transferred to Whatman P-81 paper by the Southern technique [15]. The paper was washed several times with distilled water at 80°C, then at room temperature, and dried before being exposed to MAFE RP-X1 film for autoradiography, using an amplifying screen.

RESULTS AND DISCUSSION

Purification of the HPH activity

Initial attempts to displace HPH from the Sepharose-6B–hygromycin-B column by using high concentrations of (NH₄)₂SO₄ (up to 2 M) were unsuccessful. This has also been found for other aminocyclitol-modifying enzymes [9]. However, the HPH enzyme was eluted with a solution of hygromycin B, as described in Materials and Methods. A summary of the purification of HPH is given in Table 1. After the Sepharose-6B–hygromycin-B affinity chromatography step (Fig. 1) and removal of contaminating hygromycin B, a 236-fold purification was achieved. Only 10.8% of the total HPH activity estimated in the 100000 \times g supernatant was recovered from the column. This low value might be explained by a partial inactivation of the enzyme during the elimination of the hygromycin B from the active column fractions (Table 1). Alternatively, this loss may represent an inactivation on the column or a lack of recovery from it. In the final preparation one unit of HPH activity corresponded to 2 μg HPH enzyme.

The analysis of the purified preparation by SDS-PAGE showed only a single protein band with an apparent molecular mass of 41 kDa (Fig. 2). This value is in good agreement with that previously found (42 kDa) by gel filtration of HPH [8].

Apparently, the HPH purified by this procedure has an heterogeneous amino terminal: Thr-Gln-Glu-Ser-Leu-Leu or Ser-Leu-Leu-Leu-Leu-Asp, indicating that, after translation, it is processed [16].

The HPH activity was stabilized in the presence of 0.1% bovine serum albumin and 50% (v/v) glycerol. Under these conditions it could be kept at –20°C for up to six months with no apparent loss of activity.

Detection of HPH activity in polyacrylamide gels

To confirm that the purified HPH activity corresponded to the single protein band detected by SDS-PAGE (Fig. 2), an *in situ* phosphorylation reaction was performed. Duplicate 3- μg samples of purified HPH enzyme, were separated by 7.5% PAGE in the absence of SDS. The gel was then divided into halves. One was stained, and the other was used to perform the phosphorylation reaction as described in Materials and Methods. The synthesized 7'-O-phosphoryl-hygromycin B [8] was transferred to phosphocellulose paper, which was then exposed to a X-ray film. Only one spot of radioactivity, coincident with the protein band detected in the parallel gel, was apparent (Fig. 3). The finding of a diffuse spot of radioactivity was probably due to diffusion of the phosphoryl-hygromycin B in the agarose gel. These observations strongly suggested that the purified protein of 41 kDa was HPH.

Biochemical analysis of HPH enzyme

The HPH activity was absolutely dependent on magnesium, with an optimum at 100 μM , and had an optimum pH of 7.5 (Fig. 4). In addition, a substrate inhibition was found at hygromycin B concentrations higher than 10 μM .

Due to the high affinity of HPH for its antibiotic substrate, the spectrophotometric assay described by Thompson et al. [17] could not be applied to determine the K_m values for hygromycin B and ATP because of its lack of sensitivity. Therefore, the radiochemical assay, a much more sensitive method than the spectrophotometric assay, was used although this technique had the drawback of some non-linearity with respect to time and enzyme concentration [9]. The results were plotted (Fig. 4) and the K_m values for hygromycin B and ATP were deduced by applying the least-squares technique. Approximate K_m values of 0.56 μM (linear regression

coefficient, $r = 0.999$) and $36.4 \mu\text{M}$ ($r = 0.984$) were calculated for hygromycin B and ATP, respectively from a Lineweaver-Burk double-reciprocal plot. These values are close to those found previously with a partially purified preparation of HPH [8]. The K_m value of HPH for hygromycin B is one of the lowest found for aminocyclitol-modifying enzymes [4, 10, 17–19].

The method described in this work to purify HPH enzyme seems to be of general use for a variety of aminocyclitol-modifying enzymes since we have successfully used it to purify three different hygromycin B phosphotransferases from other *Streptomyces* sp. (D. Abarca, A. Bárcena, M. Z. and A. J., unpublished results), as well as the HPH produced by an *Escherichia coli* clone carrying the *hyg* gene (see preceding paper [20]). Moreover, a Sepharose-6B–paromomycin column chromatography step allowed the preparation of a highly purified paromomycin phosphotransferase and a paromomycin acetyltransferase from *Streptomyces lividans* clones containing the relevant genes from *Streptomyces rimosus f. paromomicinus* (J. A. Pérez-González, M. López, D. Mateos, J. M. Pardo and A. Jiménez, unpublished results).

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