

# Cloning, purification and characterisation of a recombinant purine nucleoside phosphorylase from *Bacillus halodurans* Alk36

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**Abstract** A purine nucleoside phosphorylase from the alkaliphile *Bacillus halodurans* Alk36 was cloned and overexpressed in *Escherichia coli*. The enzyme was purified fivefold by membrane filtration and ion exchange. The purified enzyme had a  $V_{\max}$  of  $2.03 \times 10^{-9} \text{ s}^{-1}$  and a  $K_m$  of 206  $\mu\text{M}$  on guanosine. The optimal pH range was between 5.7 and 8.4 with a maximum at pH 7.0. The optimal temperature for activity was 70°C and the enzyme had a half life at 60°C of 20.8 h.

**Keywords** Nucleoside phosphorylase · Biocatalysis · Guanosine · 5-Methyluridine · *Bacillus halodurans*

## Abbreviation

BHPNP1 *Bacillus halodurans* purine nucleoside phosphorylase 1

## Introduction

5-Methyluridine is a non-natural nucleoside that can be used as an intermediate in the synthesis of thymidine, and

in the synthesis of nucleoside analogues AZT and stavudine, both of which are used in highly active antiretroviral treatment of HIV/AIDS patients. As the compound needs to be formed as a single isomer, 5-methyluridine can be synthesised through the transglycosylation of D-ribose-1-phosphate, using guanosine as a donor, and thymine as receptor (Ge et al. 2009; Medici et al. 2008; Rocchietti et al. 2004). Enzymes provide regio- and stereoselectivity, and hence are an ideal option for nucleoside transglycosylation (Prasad et al. 1999; Utagawa 1999). The hydrolysis reaction for the ribose decoupling reaction can be achieved using purine nucleoside phosphorylase (PNPase; EC 2.4.2.1) (Fig. 1).

However, the reagents guanosine and thymine are relatively insoluble, and are particulate substrates with poor reaction kinetics. The most effective method of solubilising these materials is in hot aqueous solutions. Therefore, it would be preferable to utilise thermostable enzymes to catalyse these reactions. Enzymes that can be used in this transglycosylation reaction include PNPase, thymidine phosphorylase (TPase; EC 2.4.2.4) and uridine phosphorylase (UPase; EC 2.4.2.3) (Bzowska et al. 2000; Pugmire and Ealick 2002). TPase and UPase are functionally both pyrimidine nucleoside phosphorylases (PyNPase; EC 2.4.2.2.), although UPase is closer in sequence identity to PNPase than PyNPase (Lewkowicz and Iribarren 2006). PNPases are divided into two different classes depending on their tertiary and quaternary structures (Bzowska et al. 2000). Type I PNPases tend to be bacterial in origin and, based on sequence and structural information, appear to have a hexameric structure. Type II PNPases tend to be found in eukaryotes and, based on the sequence and structural information, are trimeric in structure (Bzowska et al. 2000; Pugmire and Ealick 2002). Bzowska et al. (2000) refer to type I PNPases as high molecular mass

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## DNA sequencing

The insert was sequenced at Inqaba Biotechnology (Pretoria, South Africa) using the PCR primers described above. The sequence was compared with the known nucleotide and amino acid sequence of the BH1531 gene from *B. halodurans* C-125 (protein sequence—BAB05250) and has been submitted to GenBank under the accession number GQ390428.

## Homology modelling

Multiple sequence alignments were performed using ClustalW (Larkin et al. 2007). Homology modelling was performed using Accelrys Discovery Studio 2.0. A trimeric model was based on the bovine structure 1LVU (Bzowska et al. 2004). A second model was based on the monomeric structure 1VFN (Koellner et al. 1997). Bovine PNPase and BHPNP1 have 49% sequence identity and 61% sequence similarity (Table 1).

## Growth and induction

Recombinant *E. coli* strains were grown in 50 ml LB medium with 100 µg/ml ampicillin, at 37°C with shaking at 200 rpm. Cultures were induced with 0.25 mM IPTG when they had reached an OD<sub>600</sub> between 0.05 and 0.1. Cultures were subsequently grown at 30°C with shaking at 150 rpm overnight for enzyme expression.

## Batch fermentations

A 1.5 l InFors HT batch fermentor (Labfors, Switzerland) containing 1 l of GMO 20 medium was inoculated with a 50 ml inoculum (overnight culture of *E. coli* JM109 [pMSPNP] in LB medium). The composition of the GMO 20 medium was as follows: 14.6 g/l K<sub>2</sub>HPO<sub>4</sub>, 2 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3.6 g/l Na<sub>2</sub>HPO<sub>4</sub>, 2.5 g/l citric acid, 1.2 g/l MgSO<sub>4</sub>, 5 g/l NH<sub>4</sub>NO<sub>3</sub>, and 20 g/l yeast extract. Glucose (17.5 g/l) and trace element solution (5 ml/l) was sterilized separately and added to the fermenters before inoculation.

Ampicillin (100 µg/ml) was aseptically added to the flasks containing the glucose and trace element solution. The trace element solution consisted of the following: 0.4 g/l CaCl<sub>2</sub>·2H<sub>2</sub>O, 16.7 g/l FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.15 g/l MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.18 g/l ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.125 g/l CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.18 g/l CoCl<sub>2</sub>·6H<sub>2</sub>O, and 20.1 g/l Na<sub>2</sub>EDTA. The pH of the fermentations was controlled at pH 7.2 with 33% *m/v* NH<sub>4</sub>OH or 20% *m/v* H<sub>2</sub>SO<sub>4</sub>. The temperature was controlled at 37°C and the aeration set to 1 *v/v/m*. The starting agitation was set at 300 rpm and ramped up manually to control the pO<sub>2</sub> above 30% saturation. PNPase expression was induced at mid-log phase by adding IPTG to a final broth concentration of 0.5 mM. Fermentations were run for a further 4 h after induction.

## Preparation of crude extract

After induction, the bacteria were harvested by centrifugation (15,000g, 20 min). The pellet was re-suspended in minimal sterile deionised water, and subjected to a freeze–thaw cycle alternating between +20 and –20°C. Liberated protein was separated by centrifugation (14,000g, 10 min) and stored. The pellet was re-suspended in 1 l sterile deionised water and further disrupted using a cell disruptor (2 Plus, Constant Systems, UK) with 1 pass at 40 kpsi. Cellular debris was removed by centrifugation (15,000g, 10 min). The resultant protein solution (supernatants from freeze–thaw and cell disruption processes) were concentrated and washed once with sterile deionised water by ultrafiltration using an Amicon (Millipore, USA) stirred cell ultrafiltration unit (30 kDa cut-off polyethersulfone membrane). The final preparation was lyophilised in the presence of 1% maltose and 1% PEG 8000 (Vertis Genesis 25 l). A portion of the lyophilised product, equivalent to 200 ml original fermentation broth, was re-suspended in 20 mM Tris–HCl buffer (pH 7.5) for further purification.

## Column chromatography

Anion exchange chromatography of each sample was performed on an AKTA Prime (Amersham Biosciences,

**Table 1** Comparison of various PNPases to BHPNP1

Protein	Percentage of protein identity	Percentage of protein similarity	PNPase type	Accession number
<i>E. coli</i> PNP	17.0	33.0	I	P0ABP8
<i>E. coli</i> XapA	44.0	61.0	II	NP_416902
<i>G. stearothermophilus</i> PNP1	74.9	86.2	II	P77834
Bovine PNP	47.1	61.1	II	P55859
Human PNP	45.2	58.5	II	P00491
<i>B. halodurans</i> BH1532	57.1	75.6	II	BAB05251
<i>B. subtilis</i> PNP	69.6	78.8	II	P46354
<i>G. stearothermophilus</i> PNP2	18.3	32.2	I	P77835

UK) using Toyopearl SuperQ650m anion exchange resin (Tosoh BioSep, USA). Protein was first eluted from the column using a salt gradient of between 50 and 500 mM NaCl in 20 mM Tris–HCl pH 7.2, over 400 ml at a flow rate of 4 ml/min. PNPase activity was assayed in all fractions (5 ml fractions collected) and those containing activity were separately pooled and concentrated by ultrafiltration (30 kDa membrane, Millipore USA). This sample was then re-applied to the anion exchange column and eluted over a salt gradient of 150–400 mM NaCl. Active fractions were pooled and concentrated by ultrafiltration as above.

### Tertiary conformation

Denatured (5 min, 95°C) and non-denatured preparations of the enzyme were analysed on a 12% SDS-PAGE gel. The gel was overlaid with a 0.5% agarose solution to determine the position of active subunits. The agarose solution contained 10 mM inosine, 0.2 U/ml xanthine oxidase and 10 mM INT (iodonitrotetrazolium violet) in 20 mM sodium phosphate buffer, pH 7.5. Active PNPase is indicated by a red/pink band on the gel due to the cascade action of the PNPase and xanthine oxidase leading to the reduction of INT to its tetrazolium salt. Positions of active and non-active units were then confirmed by staining the gel with Coomassie brilliant blue G-250.

### PNPase assay

A volume (10 µl) of suitably diluted sample was added to 190 µl of 50 mM sodium phosphate buffer containing 0.5 mM inosine and 0.2 U/ml of xanthine oxidase in UV compatible microtitre plates (Thermomix) (Erion et al. 1997). The change in absorbance at 293 nm due to the liberation of uric acid was measured on a Powerwave HT microplate spectrophotometer (Biotek, USA). One unit of PNPase is defined as the amount of enzyme required to liberate 1 µmol of uric acid from inosine, in the presence of an excess of xanthine oxidase, in 1 min. The extinction coefficient ( $\epsilon$ ) under these conditions was determined to be 7,454 cm<sup>2</sup>/mol.

### Physical characteristics

A pH profile was performed using reaction mixtures (1 ml) containing 1 mM guanosine in 50 mM universal buffer (50 mM Tris, 50 mM boric acid, 33 mM citric acid 50 mM Na<sub>2</sub>PO<sub>4</sub>), adjusted to pH values between 3 and 11 with either HCl or NaOH. PNPase (0.025 U) was added to initiate the reaction. After 10 min of incubation at 40°C, the reaction was stopped by the addition of 0.5 ml of a 5 M NaOH solution. Guanosine conversion and guanine

formation were analysed by HPLC on a Waters 2690 HPLC (interfaced with Waters Millennium Software) equipped with Waters 996 Photodiode Array Detector at 260 nm and a Phenomenex Synergi 4u Max-RP 80A, 150 × 4.60 mm column at 22°C. The mobile phase was 25 mM ammonium acetate (pH 4.0), at a flow rate of 1.0 ml/min.

The temperature optimum was determined with reaction mixtures (1 ml) containing 1 mM guanosine in 50 mM sodium phosphate buffer, pH 8.0. PNPase (0.025 U) was added to initiate the reaction. After 10 min of incubation at temperatures between 30°C and 90°C, the reaction was stopped by the addition of 0.5 ml of a 5 M NaOH solution. Guanosine conversion and guanine formation were analysed by HPLC as above. For temperature stability, enzyme solutions were incubated at temperatures between 40 and 70°C. Samples were removed and analysed every 30 min for the first 2 h, followed by less frequent sampling for a further 18 h.

### Kinetic parameters

The kinetic parameters for PNPase were determined for both inosine (standard assay) and guanosine (assay as described for temperature optimum study) as starting substrates. Initial substrate concentrations were varied between 0.05 and 1.0 mM. The reaction was stopped at 1, 2, 3, 4, 6 and 10 min to ensure measurements remained in the linear range. Michaelis–Menten plots and the linear transformations (Lineweaver–Burk, Hanes–Woolf and Eadie–Hofstee) were used to determine kinetic parameters.

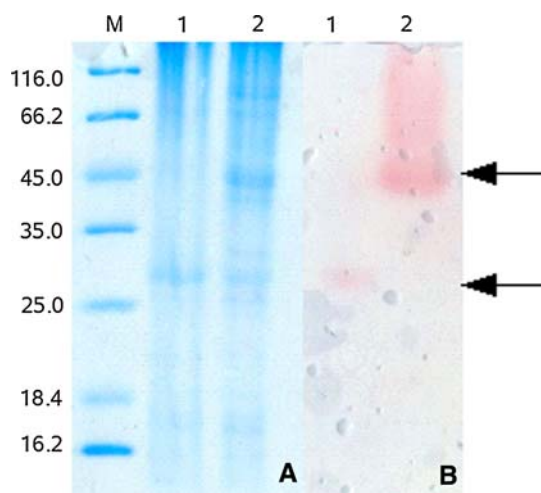
## Results and discussion

### Sequence analysis and homology modelling

*Bacillus halodurans* is unusual amongst the *Bacilli* that have been completely sequenced so far in that it contains two type II PNPases as opposed to the types I and II PNPases present in other *Bacillus* species (unpublished data). The gene sequence of BHPNP1 was identical to that of BH1531 from *B. halodurans* C-125 except for a silent substitution at nucleotide 519 (C–T), and hence the protein sequence was identical to that expressed by *B. halodurans* C-125. BLAST analysis indicated that the closest related structure deposited in the protein data base (PDB) was that of the bovine PNP (Table 1; Fig. 2) which is 47% identical to BHPNP1. On the basis of sequence identity, BH1531 is a member of the type II PNPases.

BHPNP1 has low levels of identity to type I PNPases, such as *E. coli* PNP and *G. stearothermophilus* PNP2 (17 and 18.3% identity, respectively). It has higher levels of identity to type II PNPases. Selected type II PNPases were





**Fig. 4** 12% SDS PAGE gel (a) and corresponding activity gel overlay (b). *M* marker, lane 1 heat-treated PNPase preparation (95°C, 5 min); lane 2 non-heat-treated PNPase preparation. Arrows indicate the position of the monomer and the dimer

an apparent molecular weight of 68,000, potentially indicating a dimeric protein (Hori et al. 1989b).

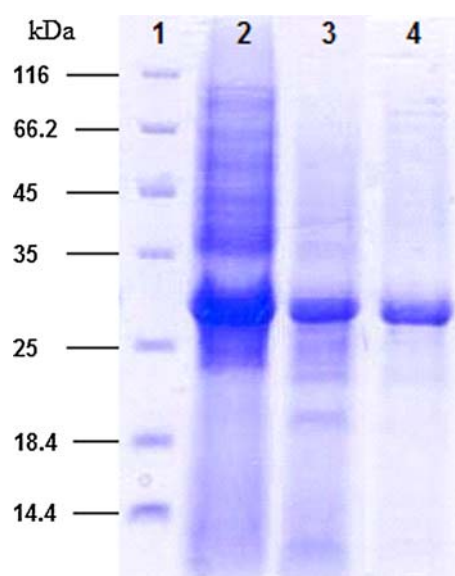
To confirm whether BHPNP1 is a trimer, which the sequence data suggests, or a dimer based on information about *G. stearothermophilus* PNP1 an overlay experiment was performed. The stained gel and overlay are shown in Fig. 4. The experiment indicated that the predominant tertiary confirmation was a dimer. The expected monomeric subunit was visible in both the heat-treated and non-heat-treated samples at approximately 27 kDa. Another dominant band at approximately 50 kDa on the Coomassie stained gel (lane 2, Fig. 4a) was related to the active band on the overlay (lane 2, Fig. 4b). Hence, in contrast to the mammalian system, this enzyme appears to be dimeric.

#### Enzyme expression and purification

The productivity of *B. halodurans* Alk36 BHPNP1 heterologously expressed in *E. coli* JM109 (DE3) was 700 U/l/h in shake flasks, but increased to 3,007 U/l/h under the controlled fermentation conditions. PNPase was purified to 42% purity (by density analysis in SDS-PAGE, Fig. 5) and a specific activity of 30.2 U/mg total protein with a fold purification of 5.0 from the culture broth (Table 2).

#### Physical characteristics

PNPase showed a pH optimum of 7.0, retaining 60% activity between pH 5.7 and 7.4 (Fig. 6). PNPase had optimum activity at 70°C and a broad activity range, retaining 60% activity between 30 and 74°C (Fig. 7). Although the optimum temperature of PNPase was shown to be 70°C, only 7.2% activity remained after 30 min



**Fig. 5** Denaturing SDS-PAGE gel (12%) showing successive purification steps. Various fractions from a purification of BHPNP were resolved using 12% SDS-PAGE. Lane 1 protein marker sizes in kilodaltons, lane 2 crude extract, lane 3 concentrated sample after anion exchange column 1, lane 4 final sample after anion exchange column 2

incubation at this temperature ( $t_{1/2}$  – 15.2 min). PNPase did, however, show good stability at 60°C ( $t_{1/2}$  – 20.8 h) and excellent stability at 40°C, with no change in activity over the time period (19 h).

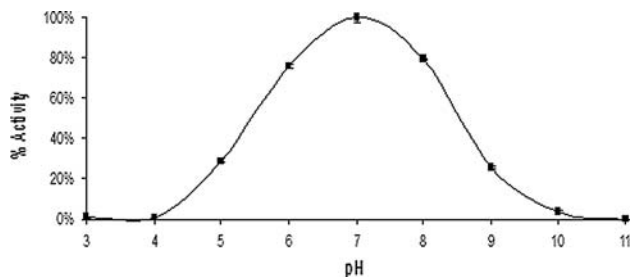
#### Kinetic characterisation

Linear transformation of velocity data obtained for varying initial substrate concentrations showed good linear regression fit where inosine was used ( $R^2 > 99\%$  for all plots) and adequate fit for guanosine experiments ( $R^2 > 94\%$ ). From the plots (Lineweaver–Burk, Eadie–Hofstee and Hanes–Wolf),  $K_m$  and  $V_{max}$  were determined with <7% deviation in the values calculated from the three plots. Subsequently, the turnover number ( $k_{cat}$ ) and the specificity constant were calculated. These values are summarised in Table 3.

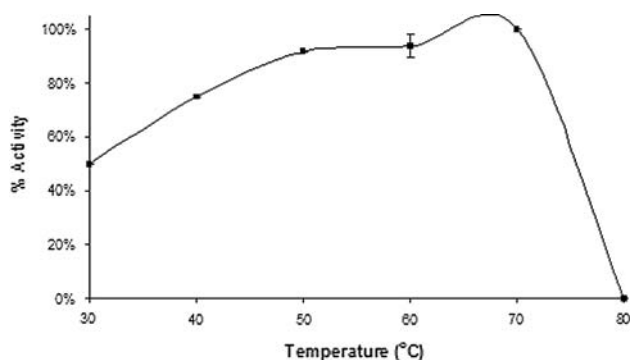
The PNP1 from the thermophile *G. stearothermophilus* has been previously expressed and characterised (Hori et al. 1989a; Hamamoto et al. 1997a). The *G. stearothermophilus* enzyme has a *pI* of 4.7, with an optimal pH range between 7.5 and 11, in contrast to the optimal pH range of BHPNP1 of between 5.7 and 8.4 and a predicted *pI* of 5.1. The *G. stearothermophilus* PNPase is more thermostable than BHPNP1 as it is stable at 70°C for greater than 30 h. In contrast, the half life of PNPase BHPNP1 at 60°C is 20 h. *G. stearothermophilus* PNPase  $K_m$  for inosine was similar at 0.22 mM, but had a greater affinity for guanosine at  $K_m$  of 0.14 mM.

**Table 2** Fold purification table for the purification of recombinant BHPNP1

	Total units	Specific activity	Percentage of recovery (%)	Fold purification
Initial culture (intracellular)	4,840	6.93	100.00	1.00
Lyophilised crude extract	732	20.65	15.12	2.97
Ion exchange 1 (0–500 mM NaCl gradient)	541	22.24	11.17	3.73
Ion exchange 2 (150–400 mM NaCl gradient)	110	30.23	2.27	4.99



**Fig. 6** pH optimum profiles of BHPNP1. A pH profile was performed using reaction mixtures (1 ml) containing 1 mM guanosine in 50 mM universal buffer (50 mM Tris, 50 mM boric acid, 33 mM citric acid; 50 mM Na<sub>2</sub>PO<sub>4</sub>), adjusted to pH values between 3 and 11 with either HCl or NaOH. PNPase (0.025 U) was added to initiate the reaction. After 10 min of incubation at 40°C, the reaction was stopped by the addition of 0.5 ml of a 5 M NaOH solution. Guanosine conversion and guanine formation were analysed by HPLC at 260 nm. The mobile phase was 25 mM ammonium acetate (pH 4.0), at a flow rate of 1.0 ml/min



**Fig. 7** Temperature optimum profile of BHPNP1. The temperature optimum of BHPNP1 was determined with reaction mixtures containing 1 mM guanosine in 50 mM sodium phosphate buffer, pH 8.0. PNPase (0.025 U) was added to initiate the reaction. After 10 min of incubation at temperatures between 30 and 90°C, the reaction was stopped by the addition of 0.5 ml of a 5 M NaOH solution. Guanosine conversion and guanine formation were analysed by HPLC

## Conclusions

The thermostable type II nucleoside phosphorylase from the bacterium *B. halodurans* Alk36 was expressed heterologously in *E. coli*, purified, and functionally characterised.

**Table 3** Physical and kinetic characteristics of BHPNP1

Parameter (units)	Inosine	Guanosine
Specific activity (U mg <sup>-1</sup> )	30.23	ND
$K_m$ (μM)	236	206
$V_{max}$ (mol s <sup>-1</sup> )	$4.76 \times 10^{-6}$	$2.03 \times 10^{-9}$
$k_{cat}$ (s <sup>-1</sup> )	$2.8 \times 10^4$	$1.2 \times 10^1$
Specificity constant (M <sup>-1</sup> s <sup>-1</sup> )	$1.2 \times 10^8$	$5.87 \times 10^4$
pH Opt	7	
pH range (60%)	5.7–8.4	
Temp Opt (°C)	70	
Temp range (60%) (°C)	32–74	
Temp stability ( $t_{1/2}$ at 60°C) (h)	20.8	
Temp stability ( $t_{1/2}$ at 40°C) (h)	>200	

The enzyme was capable of phosphorolysis of guanosine to yield guanine and ribose-1-phosphate, the latter of which may be used in the enzymatic glycosylation of nucleosides.

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