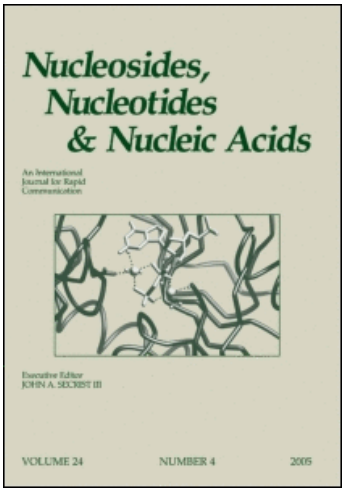

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Cloning, Expression, Purification, and Some Properties of Calf Purine Nucleoside Phosphorylase

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CLONING, EXPRESSION, PURIFICATION, AND SOME PROPERTIES OF CALF PURINE NUCLEOSIDE PHOSPHORYLASE

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□ *Calf spleen purine nucleoside phosphorylase (PNP) is considered a model enzyme for the trimeric PNPs subfamily. PCR amplification of the calf phosphorylase from the calf spleen library, cloning, overexpression of the recombinant PNP, its enzymatic activity and interactions with typical ligands of mammalian wild type PNP are described. Relative activity of the recombinant phosphorylase versus several substrates is similar to the respective values obtained for the enzyme isolated from calf spleen. As for the nonrecombinant calf PNP, the unusual fluorescence properties of the PNP/guanine complex were observed and characterized.*

Keywords Calf spleen PNP; PCR amplification; Mammalian wild type PNP

INTRODUCTION

Purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1) is a ubiquitous enzyme of the purine salvage metabolic pathway. Depending on the enzyme source, PNP is either trimeric and accepts only 6-ketopurine nucleosides as substrates, or is hexameric and shows much broader substrate specificity. Both classes of PNP are important targets for chemotherapy and also are

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interesting model enzymes for studying nonclassical enzyme kinetics due to the non-Michaelis characteristics of the PNP-catalyzed reaction.[1,2]

Calf spleen PNP is considered a model enzyme for the trimeric PNPs subfamily. Up to now only the protein isolated from calf tissue was used in these studies.[3,4] Since the molecular mechanism of the reaction catalyzed by PNP is still not fully elucidated, mutants of the model enzyme are of great interest. Therefore, we have established conditions for expressing soluble recombinant calf PNP in *E. coli* cells.

MATERIALS AND METHODS

The four primers were designed using the AF529136 sequence from the EMBL GenBank DDBJ (submitted 12-JUL-2002): A) 5′ TCGT **GGATCC** TC *ATG* CAG AAT GGA TAT ACA TAT GAA GAT 3′, B) 5′ TCGT **AAGCTT** *TTA*CAC TGG AAT GCT AGC CAT AAG AAG 3′, C) 5′ TCGT **TCTAGA** AATTTTGTTTAACTTTAAGAAGGAGATATA CAT *ATG* CAG AAT GGA TAT ACA TAT GAA GAT 3′, D) 5′ TCGT **CTCGAG** *TTA* C *TTA* C *TTA* CAC TGG AAT GCT AGC CAT AAG AAG 3′. The restriction sites for BamHI, HindIII, XbaI, and XhoI, respectively, are shown in bold. The sequence matching to *pnp* cDNA is underlined, and start and stop codons are marked in italic. All PCR reactions were run with the Platinum Pfx DNA polymerase (Invitrogen) under standard conditions. The PCR and digestion products were purified by the agarose gel electrophoresis using QIAEX II Gel Extraction Kit (Qiagen). The restriction enzymes were purchased from Fermentas. The primers A and B with the calf spleen cDNA library (Uni Zap, XR premade library) (Stratagene) were used for the first PCR reaction. The product and pQE 32 vector (Qiagen) were digested with BamHI and HindIII, ligated and transformed into *E. coli* XL1-Blue (Stratagene). The second PCR reaction was run on the pQE32::*pnp* plasmid with primers C and D. After digestion by XbaI and XhoI the product was ligated into pET28a(+) (Novagen) vector (previously also digested) and transformed into *E. coli* XL1-Blue. The recombinant plasmid was isolated and sequenced in the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics (Warsaw) and transformed into *E. coli* expression strain BL21(DE3) (Novagen). A single colony of the transformed cells was inoculated into LB medium containing 25 μg/ml kanamycin and grown overnight at 37°C. The cells were then harvested by centrifugation, suspended in 50 mM Tris-HCl (pH 7.6) and incubated at 4°C in the presence of lysozyme (0.2 mg/ml). The suspension was sonicated and centrifuged at 4°C. The supernatant was incubated with 1% (w/v) of streptomycin sulfate, centrifuged and dialyzed overnight against 50 mM Tris/HCl pH 7.6 at 4°C. The solution was then centrifuged and loaded on the FPLC Mono Q HR 5/5 column (Pharmacia). The protein was eluted

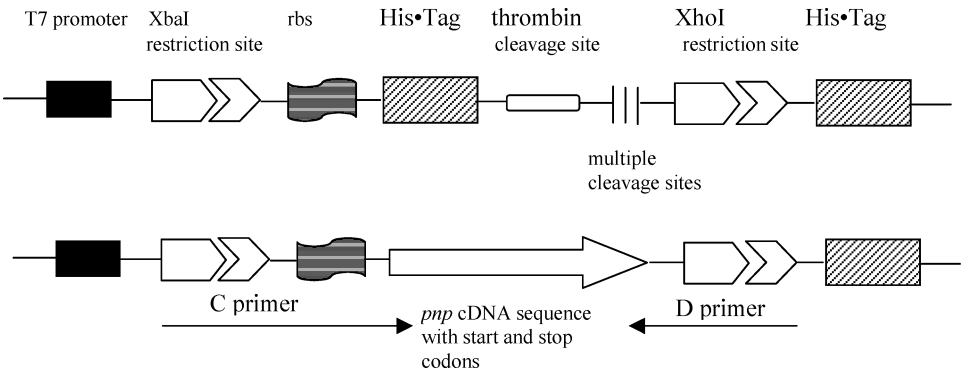

FIGURE 1 The original pET28(+) cloning/expression region (the top scheme), and the corresponding region of the pET28(+)::*pnp* construct (the bottom scheme).

with a gradient 0–100% of 0.6 M NaCl in 50 mM Tris/HCl pH 7.6. The fractions where PNP activity was detected (with 18–20%, i.e., 100–120 mM NaCl) were collected and concentrated using Amicon filters with a 30 kDa cut-off.

Protein concentration was determined by the Bradford method.[5] PNP activity was determined and all kinetic measurements were performed as earlier described.[2] The steady-state fluorescence titrations were performed at 25°C, pH 7.0. Excitation was at 290 nm and changes of the fluorescence intensity due to ligand binding were monitored at 340 nm. Mass spectroscopy analysis was performed in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics (Warsaw). The mass spectrometry data was used for identification of proteins using the Mascot program (http://www.matrixscience.com).[6] PNP purity was calculated from the SDS-PAGE gels with the TotalLab program (http://www.totallab.com).

RESULTS AND DISCUSSION

The sequence coding for PNP was amplified from the calf spleen cDNA library by the PCR method and the plasmid for the expression of the recombinant PNP in bacterial cells was constructed. The plasmid was designed to express recombinant PNP without any additional sequence tags. Nucleotide sequencing confirmed that there were no mutations in the obtained plasmid pET28a(+)::*pnp*

The recombinant protein was expressed in *E.coli* BL21(DE3) without any additional induction (e.g., by IPTG). The culture of the *E. coli* BL21(DE3) host with pET28a(+)::*pnp* was grown for 20 hours. The total protein concentration in the 10 ml of crude cell extract from 200 ml of culture was found to be about 2 mg/ml, whereas the PNP activity was approximately 14.5 U/mg. These data, when compared with the maximum activity observed for calf spleen PNP–34 U/mg, yielded the PNP concentration of 0.85 mg/ml. The

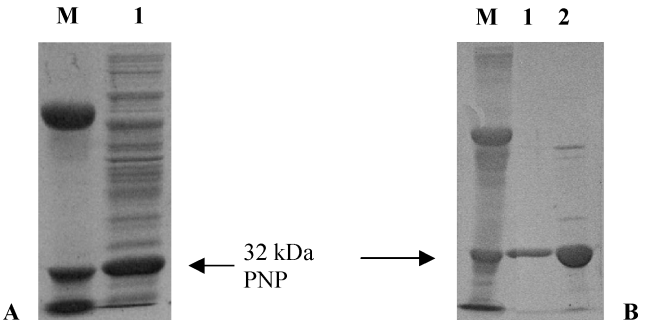

FIGURE 2 SDS-PAGE electrophoresis. Lines "M" are marker proteins 14, 29, and 63 kDa, respectively. A) line 1: crude cell extract of *E. coli* BL21(DE3) carrying pET28(+)::*pnp* plasmid after 20 hours of culturing B) line 1: single fraction with PNP activity collected after FLPC ion-exchange purification step; line 2: the final PNP product reveals 85% purity.

SDS-PAGE confirmed overexpression of a protein with the subunit molecular mass of about 32 kDa, which is in agreement with the molecular mass of the calf spleen PNP subunit (Figure 2A).

The recombinant PNP was purified using the ion exchange chromatography as described in the previous section. No significant loss of the protein or its activity was observed during the purification steps, including centrifugation, incubations, and dialysis. The PNP activity measurement conducted for all fractions obtained after the chromatography step showed that PNP does not lose more than 10% of the activity during the ion exchange step of purification and almost all protein is eluted from the column. Therefore, the production of this method yields about 40 mg of PNP from 1 l of the LB medium. PNP of the 85% electrophoretic purity was obtained (Figure 2). The molecular mass of the expressed protein determined by mass spectrometry is 31 654 Da, which is in good agreement with the molecular mass of 31 645.16 Da calculated for the 284 residues sequence with the use of the ProtParam program (http://www.expasy.org/tools/protparam.html). Other proteins in the sample were also identified. Based on these results we are planning to add the additional purification step using the gel filtration chromatography.

Calf spleen PNP is a homotrimer and needs proper folding and oligomerisation to establish full catalytic ability. At the end of the purification process we attained a purine nucleoside phosphorylase sample of the concentration of 2.9 mg/ml and activity of 26.5 U/mg, which let us claim that at least 90% of the overexpressed PNP is folded correctly. Relative activity vs inosine (Ino) and 7-methyl-guanosine (m^7Guo) was similar to the respective values obtained for the enzyme isolated from calf spleen. As for the nonrecombinant PNP, no activity vs adenosine was detected.

The kinetic behavior of the recombinant PNP versus Ino and m^7Guo substrates at saturating phosphate concentration (50 mM) are described

by the Michaelis-Menten model with K_m and V_{max} similar to the results obtained for the enzyme from calf spleen.[2] The data obtained for phosphate as the variable substrate with a saturating concentration of Ino (5 mM) showed discrepancies from the Michaelis-Menten model as in the case of the nonrecombinant phosphorylase.[2] By contrast, with a saturating concentration of m^7Guo (0.3 mM) kinetic data with phosphate as the variable substrate are sufficiently well described by the Michaelis-Menten model, again in perfect agreement with data obtained for the non-recombinant calf spleen PNP.[2] We also observed a unique fluorescence increase (about 70%) after forming the PNP/guanine complex. From the steady-state fluorescence titrations we have obtained the dissociation constant of 0.08 ± 0.02 μM describing binding of guanine, which is consistent with the previously reported value for the non recombinant enzyme.[7,8]

All results presented above indicate that calf spleen PNP overexpression in *E. coli* bacterial cells is an efficient and fast method to produce significant amounts of this protein. The enzyme seems to show high activity and behavior similar to the protein isolated from mammalian tissue. Thus, it can fully replace the non-recombinant PNP.

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