Expression of a functional non-ribosomal peptide synthetase module in *Escherichia coli* by coexpression with a phosphopantetheinyl transferase

Jung Ku[1], Raghavendra G Mirmira[1,2], Lu Liu[1]* and Daniel V Santi[1,3]

Background: Non-ribosomal peptide synthetases (NRPSs) found in bacteria and fungi are multifunctional enzymes that catalyze the synthesis of a variety of biologically important peptides. These enzymes are composed of modular units, each responsible for the activation of an amino acid to an aminoacyl adenylate and for the subsequent formation of an aminoacyl thioester with the sulfhydryl group of a 4′-phosphopantetheine moiety. Attempts to express these modules in *Escherichia coli* have resulted in recombinant proteins deficient in 4′-phosphopantetheine. The recent identification of a family of phosphopantetheinyl transferases (P-pant transferases) associated with NRPS have led us to investigate whether coexpression of NRPS modules with P-pant transferases in *E. coli* would lead to the incorporation of 4′-phosphopantetheine.

Results: A truncated module of gramicidin *S* synthetase, PheAT(His_6), was expressed as a His_6 fusion protein in *E. coli* with and without Gsp, the P-pant transferase associated with gramicidin *S* synthetase. Although PheAT(His_6) expressed alone in *E. coli* catalyzed Phe-AMP formation from Phe and ATP, $<1\%$ was converted to the Phe thioester. In contrast, $>80\%$ of the PheAT(His_6) that was coexpressed with Gsp could form the Phe thioester in the presence of Phe and ATP.

Conclusions: Our finding indicates the presence of an almost equimolar amount of 4′-phosphopantetheine covalently bound to the NRPS module PheAT(His_6), and that the functional expression of NRPS modules in *E. coli* is possible, provided that they are coexpressed with an appropriate P-pant transferase.

Addresses: [1]Department of Pharmaceutical Chemistry, [2]Department of Medicine and [3]Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143 0448, USA.

*Present address: Kosan Biosciences, Burlingame, CA 94010, USA.

Correspondence: Daniel V Santi
E-mail: santi@socrates.ucsf.edu

Key words: coexpression, gramicidin *S* synthetase, module, non-ribosomal peptide synthetase, phosphopantetheine

Received: **10 February 1997**
Accepted: **28 February 1997**

Electronic identifier: **1074-5521-004-00203**

Chemistry & Biology March 1997, **4**:203–207

Introduction

Non-ribosomal peptide synthetases (NRPSs) are a family of multifunctional enzymes that catalyze the biosynthesis of a variety of bioactive peptides, many of which have important pharmaceutical applications [1]. NRPSs are composed of 'modules', each of which is responsible for activation of a specific amino acid as its aminoacyl adenylate (aa-AMP), followed by thioesterification of the amino acid to a covalently bound 4′-phosphopantetheine (4′-PP) [2]. The aminoacyl moieties of the thioesters are then condensed to form peptide bonds and the final peptide is modified further by cyclization or hydrolysis.

To date, expression and mutagenesis systems for functional NRPSs have been limited to host organisms that naturally produce the bioactive substances (e.g. *Bacillus subtilis*, *Aspergillus nidulans* and *Neurospora crassa*) [3–5]. Although such systems demonstrate the feasibility of generating functional recombinant NRPSs, so far they have not provided practical sources for obtaining the quantities of recombinant enzyme needed for biochemical and structural characterization. Clearly, heterologous expression of functional NRPSs in *Escherichia coli* would be desirable because it would enable us to benefit fully from the advanced base of knowledge and technology that is associated with the use of this organism. Although NRPS modules that are capable of aa-AMP formation can be expressed in *E. coli*, they have been inefficient at the subsequent formation of the aminoacyl (aa) thioesters [6–8]. At least one reason for this is that NRPS modules expressed in *E. coli* do not contain sufficient 4′–PP cofactor, presumably because the endogenous phosphopantetheinyl transferases (P-pant transferases) are unable to recognize the heterologous NRPS modules as substrates. Recently, Lambalot *et al.* [9] identified a family of P-pant transferases and showed that they are capable of transferring the 4′-PP cofactor from coenzyme A (CoA) to NRPS modules *in vitro*.

The objective of the present work was to establish whether NRPS modules capable of significant aa-thioester formation could be obtained in *E. coli* by coexpressing a sequence encoding the module with a cognate P-pant transferase. If phosphopantetheinylation *in vivo* of component modules of NRPS can be achieved, a similar approach

might enable heterologous expression of fully functional multi-module NRPSs. We coexpressed a truncated module (residues 1–655) of the GrsA protein of gramicidin *S* synthetase, PheAT(His_6), in *E. coli* with the P-pant transferase associated with gramicidin *S* synthetase, Gsp [9]. Although <1% the PheAT(His_6) expressed alone contained 4′-PP, >80% of the PheAT(His_6) coexpressed with Gsp contained 4′-PP and could therefore form a thioester with [^{14}C]Phe.

Results and discussion

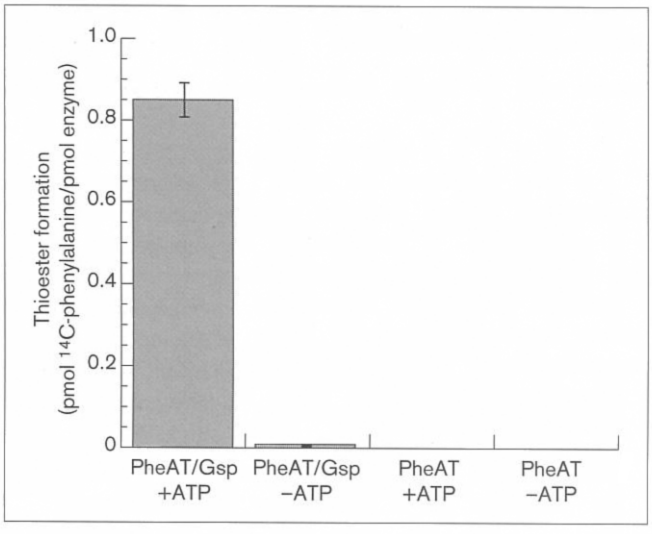
The DNA sequence encoding amino acid residues 1–655 of GrsA was amplified from *Bacillus brevis* genomic DNA by the polymerase chain reaction (PCR), and was cloned behind the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T5 promoter of the expression vector pQE60 to give pQE-PheAT(His_6). The encoded PheAT(His_6) sequence contains the putative aa-AMP-forming and thioesterification domains of GrsA followed by a six His tag (His_6) which allows simple purification using a Ni^{2+}-nitrolotriacetic acid (His-binding) resin [10].

Similarly, the gene encoding Gsp, the cognate P-pant transferase for GrsA, was amplified from *B. brevis* genomic DNA, placed behind a T5 promoter, and cloned into pDPT2789 to give pDPT-Gsp. pDPT-Gsp contains an incFII origin of replication compatible with coexpression with the ColE1 origin of pQE-PheAT(His_6), and a chloramphenicol resistance gene for selection of organisms that harbor both pDPT-Gsp and the ampicillin resistance-encoding plasmid pQE-PheAT(His_6).

Cultures of *E. coli* X90 containing pQE-PheAT(His_6) or pQE-PheAT(His_6) + pDPT-Gsp were grown at 30°C and transcription was induced with IPTG. The PheAT(His_6) from both systems was purified to >95% homogeneity by successive chromatography through Q-Sepharose, His-binding resin and hydroxylapatite. The specific activities of the enzymes in the ATP-PP_i exchange reaction were similar: 0.95 μmol min^{-1} mg^{-1} for PheAT(His_6) expressed alone and 0.97 μmol min^{-1} mg^{-1} for PheAT(His_6) coexpressed with Gsp.

The 4′-PP cofactor content of PheAT(His_6) was determined by measuring the ability of the enzyme to form a covalent complex with [^{14}C]Phe in the presence of ATP [11]. As shown in Figure 1, <1% of PheAT(His_6) expressed alone in *E. coli* was capable of forming a covalent complex with [^{14}C]Phe, as determined by quantification of acid-insoluble radioactivity. This result is similar to that observed with other NRPS modules that have been expressed in *E. coli* [6–8], and is not significantly different from that observed in the absence of ATP (Fig. 1). To show that this low level of Phe-thioester formation was not due to prior thioester formation *in vivo* that withstood conditions of purification, the preparation was treated with NH_2OH under conditions that disrupt the Phe thioester, and then re-charged with

Figure 1



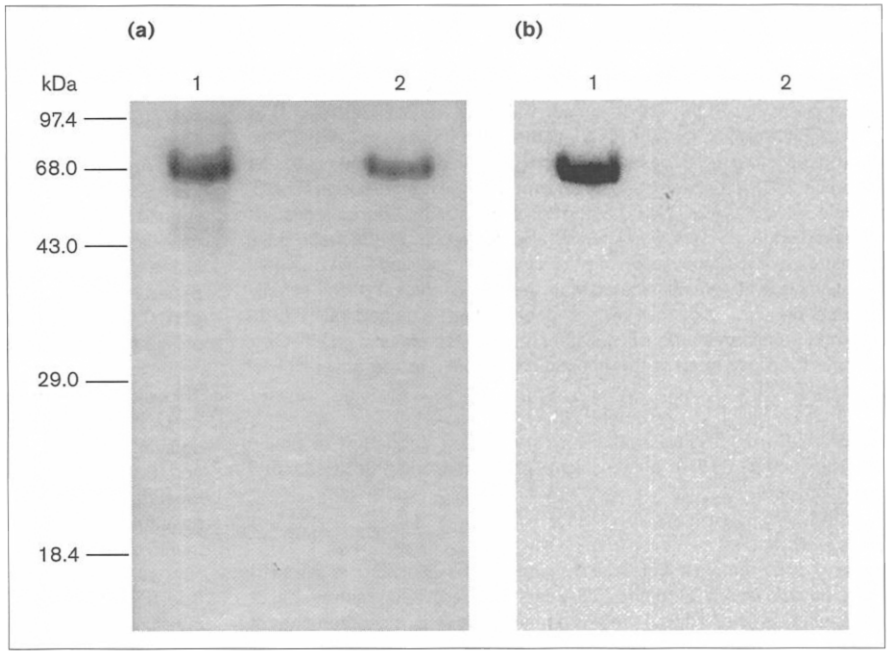
Thioester formation of PheAT(His_6) with [^{14}C]Phe. Reaction conditions are detailed in the Materials and methods section. PheAT/Gsp, PheAT(His_6) coexpressed with Gsp; PheAT, PheAT(His_6) expressed alone; +ATP and −ATP, reaction in the presence and absence of ATP, respectively. The error bar reflects standard deviations in the assay (n=8).

[^{14}C]Phe and ATP. As before, <1% of the PheAT(His_6) was found to form the thioester with Phe. Furthermore, when PheAT(His_6) expressed alone was treated *in vitro* with CoA and a crude extract containing Gsp and then Phe and ATP were added, ~40% of PheAT(His_6) was found to form a thioester with Phe. The incomplete thioester formation was attributed to the crude system used.

The 4′-PP content of PheAT(His_6) coexpressed with Gsp was also determined by measuring the ability of the enzyme to form a covalent complex with [^{14}C]Phe in the presence of ATP. When this preparation of PheAT(His_6) was treated with [^{14}C]Phe and ATP and analyzed using SDS-PAGE, radioactivity migrated with the 70 kDa protein, thus indicating the covalent modification of the enzyme by [^{14}C]Phe. In contrast, no radioactive band was detected with the PheAT(His_6) expressed in the absence of Gsp (Fig. 2). As quantified by acid insoluble radioactivity, >80% of the PheAT(His_6) coexpressed with Gsp was covalently modified with [^{14}C]Phe in an ATP-dependent reaction (Fig. 1). This finding indicates that this preparation of PheAT(His_6) contained an almost equimolar amount of the 4′-PP cofactor. Treatment of this preparation of PheAT(His_6) with NH_2OH, followed by the addition of [^{14}C]Phe and ATP resulted in Phe-thioester formation that was 86% of that observed prior to NH_2OH treatment. This result confirms that the enzyme did not contain significant amounts of amino acid thioesterified *in vivo* that withstood conditions of purification. Taken together, our results indicate that PheAT(His_6), when

Figure 2

SDS-PAGE analysis of PheAT(His_6) charged with [^{14}C]Phe. **(a)** Coomassie blue stained gel; **(b)** autoradiogram of the same gel. Lane 1, PheAT(His_6) coexpressed with Gsp; lane 2, PheAT(His_6) expressed alone. See text for details.



coexpressed with Gsp in *E. coli*, remains catalytically active and contains an almost equimolar amount of cofactor 4′-PP.

Significance

In the work described here we coexpressed a truncated module of gramicidin *S* synthetase, PheAT(His_6), with and without Gsp, an enzyme that is thought to transfer the 4′-phosphopantetheine (4′-PP) moiety from coenzyme A (CoA) to gramicidin *S* synthetase. Our results confirm previous observations that when PheAT(His_6) alone is expressed in *Escherichia coli*, the formation of a thioester with Phe is deficient because there are inadequate amounts of the covalently bound cofactor 4′-PP. In contrast, we find that when PheAT(His_6) is coexpressed with Gsp, about 85% of the protein can form a Phe thioester, indicating the presence of an almost equal amount of covalently bound 4′-PP. By using the coexpression technology described here, it may be possible to express fully functional multimodular non-ribosomal peptide synthetases (NRPSs) in *E. coli*. This is currently impossible because insufficient endogenous 4′-PP is transferred to NRPSs which need it in order to function. If it is possible to express fully functional NRPSs in *E. coli*, this would expand greatly our ability to genetically manipulate NRPS modules for peptide drug synthesis. This technology may also have significance in other systems, such as polyketide synthesis. Because polyketide synthases expressed in *E. coli* are also deficient in 4′-PP incorporation [12], it is possible that the coexpression of polyketide synthases with the appropriate P-pant transferases might also yield fully functional proteins.

Materials and methods

Materials and DNA manipulations

All enzymes and reagents were the purest grade commercially available. L-[U-^{14}C]Phe (448 mCi mmol^{-1}) and [^{32}P]PP_i (30 Ci mmol^{-1}) were from Amersham and NEN, respectively. The His-Binding kit was purchased from Novagen. Q-Sepharose was from Pharmacia. Hydroxylapatite was from Bio-Rad. DNA manipulations and PCR were performed using standard methods [13]. PCR was performed using a Perkin Elmer 9600 Gene Amp system. Automated DNA sequencing and oligonucleotide synthesis were performed at the Biomolecular Resource Center (BRC) at the University of California, San Francisco, USA.

Plasmids, bacterial strains and culture conditions

The expression vector pQE60 (including the ampicillin resistance gene, Amp^r) containing the T5 promoter under the control of two *lac* operators, a ColE1 origin of replication, and six tandem His codons at the carboxyl terminus of the coding sequence, was obtained from Qiagen. pDPT2789 (including the chloramphenicol resistance gene, Cm^r), a high copy number plasmid with an incFII origin of replication [14], was a gift from AR Schatzman (SmithKline Beecham Laboratories). *E. coli* DH5α, from Bethesda Research Laboratories, USA, was used for manipulation of recombinant plasmids. *E. coli* X90 (F′ *lacI^q*, *lacZY*, *proAB/D(lac-pro)*, *ara*, *nalA*, *argE(am)*, *thi*, *rif^r*), a gift from C Craik, was used as the expression host. *B. brevis* (ATCC9999) was obtained from ATCC. *E. coli* strains were grown in either Luria–Bertani broth (LB) (1% yeast extract, 1% tryptone, 0.5% NaCl) or Terrific broth (TB) (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 90 mM potassium phosphate, pH 7.4) at the specified temperature.

Construction of expression vectors

The *gsp* gene was obtained by PCR amplification of genomic *B. brevis* DNA. The 5′ *gsp* primer (ON1: 5′ CAT G**CC ATG G***TA GAA ATG TTA TTT GTA AAG GT* 3′) contained a 4-base spacer, an *Nco*I restriction site (bold) containing the start codon, and a 22 base sequence complementary to bases 5–26 of the *gsp* gene (italics); introduction of the *Nco*I site results in an Ile to Val mutation at codon 2. The 3′ *gsp* primer (ON2: 5′ C CC**A AGC TT***A AAA GTT ATT ATT TTC AGA AAA* 3′) contained a 3-base spacer, a *Hind*III site (bold), a new stop codon sequence (TAG

to TAA), and a 23 base sequence complementary to nucleotides 706–728 of the *gsp* gene (italics). The amplified DNA fragment was digested with *Nco*I and *Hind*III, ligated with *Nco*I/*Hind*III-digested pQE60 to give pQE-Gsp, and transformed into *E. coli* X90.

The *gsp* gene, together with the T5 expression system from pQE-Gsp, was amplified by PCR. The 5′ primer (ON3: 5′ AGG CCC **AAG CTT** *CGT CTT CAC CTC GAG AAA* 3′) contained a 6-base spacer, a *Hind*III site (bold), and an 18 base sequence (italics) complementary to the sequence 206–223 base pairs upstream of the *gsp* start codon. The 3′ primer (ON4: 5′ CAG GAG TCC AAG CTC AGC 3′) consisted of an 18 base sequence complementary to the sequence 11–28 base pairs downstream of the stop codon of pQE-Gsp. The amplified DNA fragment was digested with *Hind*III, ligated with the large fragment of the *Hind*III-digested and calf intestinal alkaline phosphatase digested pDPT2789 DNA and transformed into *E. coli* X90. The isolated plasmid, pDPT-Gsp, contained the *gsp* gene in the orientation opposite to the orientation of Cm^r of pDPT2789.

Vector pQE-PheAT(His_6) containing the coding sequence of amino acid residues 1–655 of GrsA is identical to the construct described previously [15].

Protein analysis

Protein concentrations were determined by the Bradford assay [16] using bovine serum albumin (BSA) as standard. Concentrations of homogeneous PheAT(His_6) were determined by A_{280} measurements of the enzyme in 6 M guanidine hydrochloride at pH 6.5 using a calculated ε_{280} of 8.3×10^4 $M^{-1}cm^{-1}$ [17]. The calculation was based on the presence of 4 Cys, 8 Trp and 29 Tyr residues, as determined from the deduced amino acid sequence. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using either the Pharmacia Phast Gel apparatus or the Bio-Rad Modular Mini-protean II electrophoresis system.

Protein expression and purification

For expression of PheAT(His_6), a fresh overnight culture (10 ml) from a single colony of pQE-PheAT(His_6)/*E. coli* X90 was used to inoculate 1 l of TB supplemented with 100 μg ml^{-1} ampicillin, 25 μM pantothenic acid, and 10 mM $MgCl_2$. For coexpression of PheAT(His_6) with Gsp, pQE-PheAT(His_6)/*E. coli* X90 was transformed with pDPT-Gsp, and a fresh overnight culture (10 ml) from a single colony was used to inoculate 1 l of TB supplemented with 100 μg ml^{-1} ampicillin, 25 μg ml^{-1} chloramphenicol, 25 μM pantothenic acid and 10 mM $MgCl_2$. Cultures were grown at 30°C until A_{600} reached 1. IPTG was then added to a final concentration of 0.8 mM. After incubation for an additional 6–8 h at 30°C, cells were harvested by centrifugation at $5000\times g$ for 15 min at 4°C.

PheAT(His_6) preparations were purified from 500 ml cultures of pQE-PheAT(His_6)/*E. coli* X90 or pDPT-Gsp + pQE-PheAT(His_6)/*E. coli* X90. All manipulations were carried out at 4°C. The cell pellet was resuspended in 30 ml of 20 mM Tris-HCl, pH 8.0, and subjected to two cycles of disruption in a French press (15 000 psi). Cell debris were removed by centrifugation at $30\,000\times g$ for 30 min. The supernatant was loaded onto an 1.5×7 cm Q-Sepharose column previously equilibrated with 20 mM Tris-HCl, pH 8.0. The column was washed with 70 ml of 50 mM NaCl in 20 mM Tris-HCl, pH 8.0 and protein was eluted over 100 ml with 20 mM Tris-HCl, pH 8.0 containing a linear gradient of 50–600 mM NaCl. The fractions (2.5 ml each) containing PheAT(His_6) (fractions 16–22; identified by SDS-PAGE) were combined and loaded onto an 1.5×4 cm His-Binding resin column (Novagen) which had been pre-equilibrated with buffer A (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole). The column was washed with 70 ml of buffer A and protein was eluted over 100 ml with buffer A containing a linear gradient of 5–100 mM imidazole. The fractions (2 ml each) containing PheAT(His_6) (fractions 10–17) were combined and dialyzed twice against 2 l of buffer B (10 mM potassium phosphate, pH 6.8, 0.1 mM EDTA). The dialyzed protein was loaded onto an 1.5×5.5 cm hydroxylapatite column (Bio-Rad). The column was washed with 50 ml of buffer B and protein was eluted over 100 ml with buffer B containing a linear gradient from 10 to 200 mM potassium phosphate. The fractions (2.5 ml each) containing PheAT(His_6) (fractions 13–18) were combined and dialyzed two times against 2 l of 50 mM phosphate, pH 7.4, 10 mM $MgCl_2$, 5 mM DTT and 2 mM EDTA to give ~5 mg of protein.

Enzyme assays

Aminoacyl adenylate formation was monitored by ATP–[^{32}P]PP_i exchange [18]. A solution (100 μl) containing 10 nM PheAT(His_6), 50 mM Tris-HCl, pH 7.8, 1 mM ATP, 0.1 mM PP_i, 3×10^5 cpm [^{32}P]PP_i, 1 mM $MgCl_2$, 0.1 mM EDTA and 1 mM Phe was incubated at 25°C for 15 min. The reaction was quenched by the addition of 1 ml of 1% (w/v) activated charcoal in 3% (v/v) perchloric acid. The charcoal was filtered onto glass fiber filter paper (Whatman, GF/C), washed successively with 10 ml of 0.2 M PP_i, pH 8.0, 4 ml water, 1 ml of ethanol and dried in air. Filters were placed in a scintillation vial and the radioactivity was measured by Cerenkov counting.

Thioester formation was monitored as described [11] with minor modifications. A solution (100 μl) containing PheAT(His_6) (1.0 μM), 10 mM sodium phosphate, pH 7.8, 10 mM $MgCl_2$, 2 mM ATP, 3 μM L-[U-^{14}C]Phe and 0.1 unit of pyrophosphatase was incubated at 25°C. ATP was omitted in the control. After 10 min, 10 μl of 25 mg ml^{-1} BSA was added as carrier and the protein was precipitated with 0.8 ml of 8% cold trichloroacetic acid (TCA). The precipitate was collected on a glass fiber filter paper and washed with 10 ml of cold 8% TCA. 6 ml of scintillation fluid (RPI, Biosafe II) was added to the filter and the radioactivity was measured in a scintillation counter. The time dependence of thioester formation showed that reaction was complete within 1 min. For SDS-PAGE analysis of Phe-PheAT(His_6) thioester, the above reaction mixture was treated with an equal volume of loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and subject to electrophoresis on a Bio-Rad Mini-Protean II electrophoresis system. The gel was visualized by staining with Coomassie blue, prepared for fluorography as described [19], dried and exposed to X-ray film (Kodak XAR).

NH_2OH treatment of PheAT(His_6)

In order to determine the time needed to completely remove covalently bound Phe from PheAT(His_6) using NH_2OH, PheAT(His_6) was first charged with [^{14}C]Phe by the method described above in a final volume of 600 μl; after 10 min at 25°C , NH_2OH (from a 1 M solution adjusted to pH 7.2 with NaOH) was added to a final concentration of 100 mM and 100 μl aliquots were removed after 1, 2, 4, 8, and 16 min. 10 μg BSA was added to each aliquot and the protein was precipitated by addition of TCA as described above. Precipitates were collected on glass fiber filter paper and radioactivity was measured. [^{14}C]Phe-PheAT(His_6) showed complete loss of acid insoluble radioactivity within 2 min.

For the treatment of PheAT(His_6) with NH_2OH, 10 μl of 1 M NH_2OH (adjusted to pH 7.2 with NaOH) was added to a solution (90 μl) containing 50 mM sodium phosphate, pH 7.8, 10 mM $MgCl_2$ and 10 μM PheAT(His_6). The reaction mixture was kept at 25°C for 5 min. Free NH_2OH was removed by application of the reaction mixture to a Nick column (Sephadex G-50, 1×2 cm, previously equilibrated with 1 ml of 50 mM sodium phosphate, 50 mM NaCl, 1 mM EDTA (buffer C)), washing with 0.4 ml of buffer C, and elution of the protein with 0.4 ml of buffer C.

Acknowledgements

We acknowledge Stephanie Wang and Rachel Duncan for their assistance in the preparation of plasmid pQE-PheAT(His_6) and for helpful discussions, as well as Pat Greene for her critical review of the manuscript. RGM is a Howard Hughes Medical Institute Physician Postdoctoral Fellow. We also acknowledge Chris Walsh and Ralph Lambalot for helpful discussions.

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