Different expression levels of two KgmB-His fusion proteins

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Abstract: The KgmB methylase from Streptomyces tenebrarius was expressed and purified using the QIAexpress System. Two expression vectors were made: pQEK-N, which places a (His)6 tag at the N-terminus, and pQEK-C, which places a (His)6 tag at the C-terminus of the recombinant KgmB protein. Kanamycin resistance of the E. coli cells containing either the pQEK-N or the pQEK-C recombinant plasmids confirmed the functionality of both KgmB-His fusion proteins in vivo. Interestingly, different levels of expression were observed between these two recombinant proteins. Namely, KgmB methylase with the (His)6 tag at the N-terminus showed a higher level of expression. Purification of the (His)6-tagged proteins using Ni-NTA affinity chromatography was performed under native conditions and the KgmB methylase with (His)6 tag at the N-terminus was purified to homogeneity >95 %. The recombinant KgmB protein was detected on a Western blot using anti-Sgm antibodies.

Keywords: KgmB methylase, Streptomyces tenebrarius, expression, purification.

INTRODUCTION

The kgmB gene from Streptomyces tenebrarius, producer of the nebramycin complex of antibiotics, encodes for the kanamycin-gentamicin resistant methylase, KgmB.1 The KgmB methylase modifies 16S rRNA at residue G-1405 and thus protects the bacteria against the produced antibiotics. The KgmB methylase shows a high level of homology of its amino acid sequence (54 %) with Sgm methylase2 from Micromonospora zionensis and 70 % homology with Grm methylase3 from Micromonospora purpurea.

It was shown previously that the sgm gene is autoregulated on the translational level.4 According to previous results with kgmB-lacZ gene fusions, the same model of translational autoregulation was proposed for the kgmB gene.5 The model is based on the fact that relatively few enzyme molecules are sufficient for complete modification

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of the target (i.e., 16S rRNA) and, thus, the KgmB methylase can bind to its own mRNA preventing further translation once all the ribosomes are modified.

In order to study the translational regulation of the kgmB gene in vitro (RNA gel shift and toeprinting experiments), the KgmB protein was expressed and purified using the QIAexpress System.6 In this way the recombinant proteins were tagged with 6 consecutive histidine residues, which makes them suitable for one step purification by immobilized metal affinity chromatography.7 In most cases, the (His)6 affinity tag does not interfere with the structure and function of the purified protein. However, in some reported cases, the position of the (His)6 tag on the recombinant protein influences its accessibility for purification8 or reduces its interaction with its ligand9 (DNA, RNA, antigen, etc.). For this reason, it was decided to make in parallel two expression constructs, whereby the (His)6 tag was placed on either the N- or C-terminus of the KgmB protein.

EXPERIMENTAL

Bacterial strains and culture conditions

The strain Escherichia coli NM522 (supE, thi, Δ(hsdRMS-mcrB), Δ(lac-proAB), F+ (proAB+, lacI, ΔlacZΔM15) was used.10 A Luria-Bertani broth (LB – 10 g tryptone, 5 g yeast extract and 5 g NaCl per 1 l, pH 7.4) was used as a rich medium and contained 15 g l⁻¹ agar when used as a solid medium.11 The antibiotics ampicillin and kanamycin were added at standard concentrations to the medium for bacteria harboring recombinant plasmids.

Recombinant DNA techniques

All routine DNA manipulation techniques, including plasmid preparation, restriction enzyme digestions, bacterial transformations, ligations and gel electrophoresis were performed according to Sambrook et al.12 The restriction enzymes were obtained from Pharmacia (Uppsala, Sweden) and were used according to the manufacturer’s instructions.

The 5’ and 3’ ends of the kgmB gene were modified by polymerase chain reaction (PCR) in two ways. One pair of primers, consisting of Fkgm30 (5’-GCGGATCCATGACGATGACAAAATGCCGCCCGCTCCC-3’) and Rkgm 30 (5’-CCCAAGCTTTCACGCGTTCTTCCG-3’), was designed to introduce BamHI and HindIII restriction sites (underlined) on the 5’ and 3’ ends, respectively. The second pair of primers, Fkgm 70 (5’-ACTTGCATGCGCACCCGGCTCCC-3’) and Rkgm70 (5’-CGGGATCCCAGCGTTCTTCCGCAGC-3’) introduces SphI and BamHI restriction sites, respectively. The PCR reactions were performed in a 50 μl reaction mixture (10 mM Tris-HCl pH 9, 50 mM KCl, 1.5 mM MgCl2,3% DMSO, 0.2 mM dNTP, 100 pmol of each primer, 1 U of Taq polymerase (Pharmacia) and 50 ng of plasmid DNA) under the following amplification profile: initial denaturation at 98 °C (10 min) followed by 35 cycles of 94 °C (1 min), 57 °C (1 min), 72 °C (1 min) and a final extension step at 72 °C for 10 min.

After PCR amplifications, the 100 bp PCR products bearing differently modified kgmB gene were purified from the agarose gel. The PCR product from the first PCR reaction was digested with BamHI and HindIII and ligated into the BamHI/HindIII sites of pQE-30 vectors (Qiagen). The PCR product from the second PCR reaction was digested with SphI and BamHI and ligated into the SphI/BamHI sites of pQE-70 vectors (Qiagen). The resulting constructs, pQEK-N and pQEK-C, were transformed into E. coli NM522 and the clones were selected by ampicillin and kanamycin resistance.

Time-course analysis of protein expression level

For determination of the KgmB-His expression level, small-scale E. coli cultures carrying either pQEK-N or pQEK-C were grown. 15 ml of LB medium containing 50 μg/ml ampicillin was inoculated with 0.75 ml of fresh overnight culture and incubated at 37 °C. When the optical density at 600 nm had attained 0.6, protein expression was induced by adding isopropyl-β-D-thiogalactopy-
ranoside (IPTG) to a final concentration of 1 mM. At 1 h intervals after induction, 1.5 ml culture samples were collected and the cells were harvested by centrifugation for 2 min at 15000 g. The cells were resuspended in denaturing lysis buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 8.0). The crude cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue (CBB) R 250 staining.

**Protein purification procedure**

For overproduction of the (His)₆-tagged KgmB proteins, the cells harboring either the pQEK-N or pQEK-C plasmid were grown in LB medium supplemented with 50 µg/ml ampicillin, 250 ml of medium was inoculated with 5 ml of fresh overnight culture and incubated at 37 °C until the optical density at 600 nm had attained 0.6. Protein expression was then induced by adding IPTG to a final concentration of 1 mM and the incubation was extended for an additional 4 h. The cells were harvested by centrifugation (5000xg, 15 min, +4 °C) resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) with 1 mg/ml lysozyme and lysed by two passages through a French press at 16000 psi. The cell extract was centrifuged (15000xg, 30 min, 4 °C) and the supernatant filtered through a 0.22 µm-pore-size filter and applied onto a Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) column equilibrated with lysis buffer. Chelate affinity chromatography was performed under native conditions according to the standard procedures recommended by the manufacturer (Qiagen).

Purification of the KgmB protein was verified by SDS-PAGE.

The protein concentrations were determined by the method of Bradford, with bovine serum albumin as the standard.

**Western blot analysis**

Western blot was performed as described by Burnette. Crude cell extracts of *E. coli* transformed with expression plasmids pQEK-N and pQEK-C were loaded onto SDS-PAGE (12.5 %), and the separated proteins were then transferred onto a nitrocellulose membrane using a Semi-Dry system Multiphor II (Pharmacia) for 1 h at 0.8 mA per cm² of membrane. The membrane was blocked with 5 % non-fat dried milk in washing buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05 % Tween 20) and was then subjected to immunoreaction with 500-fold-diluted rabbit immune serum containing the polyclonal anti-Sgm antibodies. The secondary antibody (goat anti-rabbit immunoglobulin G) conjugated with alkaline phosphatase (Sigma) was used at a 1:8000 dilution. The immunoblots were developed with nitro blue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) as the color substrate according to the manufacturer’s instructions (Promega).

**RESULTS AND DISCUSSION**

**Construction of the kgmB-his fusions**

For the construction of plasmids encoding the his-tagged *kgmB* gene, the 5’ and 3’ ends of this gene were modified by the PCR technique in two ways (Fig. 1). One pair of PCR primers was designed to allow insertion of the *kgmB* and BamHI/HindIII sites of plasmid pQE-30, creating an expression construct pQEK-N which places the (His)₆ tag at the N-terminus of the KgmB protein. The KgmB protein from this construct also contains five additional amino acids at the N-terminus which are the recognition site for enterokinase. Using enterokinase, the (His)₆ tag can be easily removed. Oligonucleotides for the other PCR were designed to allow in-frame insertion of the *kgmB* gene in the SphI/BamHI sites of plasmid pQE-70, creating an expression construct pQEK-C which places the (His)₆ tag at the C-terminus of the KgmB protein.
For expression of the KgmB protein, the pQEK-N and pQEK-C plasmids were transformed into *E. coli* strain NM 522 cells. Kanamycin resistance of the *E. coli* cells containing either the pQEK-N or pQEK-C recombinant plasmid confirmed the functionality of both KgmB-His fusion proteins *in vivo*, i.e., these proteins were able to methylate 16S rRNA.

**Time-course analysis of protein expression level by Western blot**

In order to determine the time interval after induction when the expression level of the KgmB-His fusion proteins reach their maximum, crude cell extracts were analyzed by SDS-PAGE followed by Western blot. Small-scale culture expression showed that there was a difference in expression level of N-His-KgmB and KgmB-C-His (Fig. 2). Also, a difference in the growth of *E. coli* cells carrying either expression vector pQEK-N or pQEK-C was observed. Namely, the generation time of *E. coli* / pQEK-C cells was 2.5 times longer than that of *E. coli* / pQEK-N cells. This indicates that the recombinant KgmB-C-His protein could be toxic for *E. coli* NM522 cells. Additionally, when analysed on a small-scale, the KgmB-C-His protein was not present in detectable concentrations on SDS-PAGE, despite the fact that the *E. coli* / pQEK-C cells were kanamycin resistant. Furthermore, this supports the hypothesis that only a few enzyme molecules are sufficient for complete modification of 16S rRNA.

For detection of the KgmB protein in the Western blot experiments, primary polyclonal antibodies to Sgm methylase were used. It was proposed that anti-Sgm
Antibodies can cross-react with KgmB protein because of the homology between KgmB methylase and Sgm methylase from *M. zionensis*. The Western blot showed that there is regular expression of N-His-KgmB with a linear increase in the concentration of the recombinant protein (Fig. 2a). In this experiment, the positive control was purified Sgm protein. On the other hand, KgmB-C-His protein was not detected at any time after the induction of *E. coli* /pQEk-C cells (Fig. 2b). The same unspecific pattern of bands was obtained as in the negative control (*E. coli* strain NM522).

**Purification of the KgmB-His proteins**

The different level of expression between the two KgmB-His recombinant proteins can also be noticed on the large scale. KgmB methylase with the (His)$_6$ tag at the N-terminus showed a higher level of expression and was purified on Ni-NTA agarose under native conditions to homogeneity >95% (Fig. 3a). In can be noticed that a large amount of the N-His-KgmB protein was sequestered into insoluble inclusion bodies, but the amount of soluble protein was sufficient for purification (Fig. 3a, lane 5). Expression of the N-His-KgmB protein gave rise to a major band on the SDS-polyacrylamide gel, corresponding to a 33 kDa protein with a (His)$_6$ tag and a recognition site for enterokinase.

The predicted molecular weight for the KgmB with the (His)$_6$ tag at the C-terminus is 32 kDa, which corresponds to the strongest protein band in the first eluted
fraction (Fig. 3b). The presence of KgmB-C-His in this fraction was also verified by Western blot (Fig. 3c). Compared to the same elution step in the purification of the N-His-KgmB protein, the protein concentration of KgmB-C-His is 100 times lower. Nevertheless, this showed that the growing of larger expression cultures can give the desired protein.

In this paper, different expression levels of two fusion recombinant proteins bearing a (His)$_6$ tag on either the N- or C-terminus is reported. A low-level expression of heterologous proteins can occur because the protein is toxic or unstable, or because the expression construct is not maintained in the cells during growth. Re-
combinant proteins with hydrophobic regions often have a toxic effect on the host cells. The only difference between N-His-KgmB and KgmB-C-His, except the position of the (His)_6 tag, is the presence of five charged amino acids which are the recognition site for enterokinase. This could change the net polarity and solubility of the KgmB protein and favourably influence the expression of N-His-KgmB.

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