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Cloning and overexpression of a cytidine 5′-monophosphate *N*-acetylneuraminic acid synthetase from *Escherichia coli*

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Abstract

The gene for cytidine 5′-monophosphate *N*-acetylneuraminic acid (CMP-NeuAc) synthetase (EC2.7.7.43) was amplified from the total DNA of *Escherichia coli* 44277 through a primer-directed polymerase chain reaction and cloned into pUC19 between the sites *Eco*RI and *Sal*I. The sequence analysis showed the cloned DNA was the same as the published *neuA* except for a codon missing for Asp230 and four base substitutions at the sites 153, 438, 618, and 687. The four base substitutions did not change the amino acid sequence. Based on the preliminary expression experiment and computer-aided analysis of the gene structure, six bases were mutated in order to achieve high-level expression. The gene mutated by PCR was cloned into the expression vector pET15b. Upon the induction of IPTG, the recombinant enzyme was shown to be 26% of the total bacterial proteins, which was 850-fold higher than that of the wild strain. Under the optimal inducing condition, the enzyme yield reached 100 U/l cell culture. Using the partially purified recombinant enzyme, the synthesis of CMP-NeuAc was carried out in 90% yield.

Keywords: CMP-NeuAc synthetase; Overexpression; *E. coli*

1. Introduction

Cytidine 5′-monophosphate *N*-acetylneuraminic acid (CMP-NeuAc) or sialic acid, as an important component of glycoproteins or glycolipids in mammalian tissues, plays a prominent role in a variety of biological processes, such as cell–cell communication, cell–matrix interaction, adhesion, maintenance of serum glycoproteins in the circulation, and protein targeting [1]. NeuAc is also thought to be critical virulence determinant as an important component of extracellular capsular polysaccharides in bacteria [2,3]. The biosynthesis of sialylated glycoconjugates catalyzed by various sialytransferases requires the activated form of NeuAc, CMP-NeuAc, as substrate. CMP-NeuAc is synthesized from CTP and NeuAc by CMP-NeuAc synthetase (EC2.7.7.43), which is found in mammalian cells as well as bacteria (CTP + NeuAc → CMP-NeuAc + PPi) [4]. In vitro, CMP-NeuAc can be synthesized enzymatically by means of a CMP-NeuAc synthetase [5]. It is evident that all compounds required, including

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enzymes, must be available in large quantities at reasonable costs.

CMP-NeuAc synthetase for the preparation of CMP-NeuAc is previously isolated from animal tissues, most commonly from bovine brain. However, animal tissues contain only small amounts of CMP-NeuAc synthetase and are difficult to obtain in large quantities with constant quality. Alternatively, CMP-NeuAc synthetases from bacteria such as *Neisseria meningitidis* and *Escherichia coli* have been used for CMP-NeuAc preparation [4,6–9]. CMP-NeuAc synthetase from *E. coli* was first reported to be purified to homogeneity by Vann et al. [10]. Subsequently, the gene encoding CMP-NeuAc synthetase was sequenced by Zapata et al. [11]. In order to increase the yield of enzyme production, the engineered CMP-NeuAc synthetase gene was constructed and overexpressed to the extent of 8–10% of the soluble *E. coli* proteins [12]. By modifying the ribosome binding site, the expression of CMP-NeuAc synthetase gene was increased 1000-fold [13].

In this report, the *E. coli* strain 44277 was identified to produce CMP-NeuAc synthetase. The gene coding for *E. coli* 44277 CMP-NeuAc synthetase was cloned and overexpressed in *E. coli* BL21(DE3)pLysS through a primer-directed polymerase chain reaction. The partially purified recombinant enzyme could be used for CMP-NeuAc preparation.

2. Experimental

2.1. Reagents

Inorganic pyrophosphatase, CTP, and *N*-acetylneuraminic acid were from Sigma. Restriction enzymes and T4 DNA ligase were from Promega; Vent DNA polymerase was from Bio Labs. T7 DNA sequencing kit was from Pharmacia. α-^{35}S-dATP was from DuPont. All other biochemical reagents were the highest quality commercially available.

2.2. Bacterial strains and vectors

E. coli strain 44277 was obtained from the China General Microbiological Culture Collection Center (CGMCC). *E. coli* DH5α was from Bethesda Research Laboratories. *E. coli* JM105 and pKK223-3 were from Pharmacia. *E. coli* BL21(DE3)pLysS and pET-15b were from Novagen. Plasmid pBV220 was a gift from Prof. Yunde Hou (Institute of Virology, Chinese Academy of Medical Sciences, Beijing, China). Unless otherwise indicated, all strains were grown at 37°C in LB medium contained 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl/l of culture medium. Growth medium was supplemented with 60 μg/ml ampicillin when required.

2.3. DNA manipulations and sequencing

All DNA manipulations were carried out using standard techniques described by Sambrook et al. [14] or by the manufacturers' instructions. Sequencing was performed by the dideoxy method of Sanger et al. [15] according to the directions of the Sequenase kit. Oligonucleotides were synthesized using a Beckman Oligo 1000 DNA synthesizer.

2.4. Enzyme assays

CMP-NeuAc synthetase activity was assayed by coupling the synthetase reaction to inorganic pyrophosphatase and monitoring the production of inorganic phosphate with malachite green reagent [16]. The standard assay mixture contained 0.1 M Tris–HCl (pH 9.0), 10 mM $MgCl_2$, 5.0 mM NeuAc, 1.5 mM CTP and 10 U of inorganic pyrophosphatase in a final volume of 0.5 ml. The reaction was initiated by the addition of approximate 5 mU of CMP-NeuAc synthetase. After incubation of reaction mixture at 25°C for 10 min, one aliquot of 30 μl was removed and added directly to 0.8 ml malachite green reagent (malachite green reagent: 3 ml of

0.045% malachite green hydrochloride and 1 ml of 4.2% ammonium molybdate dissolved in 4 N HCl were mixed, and 80 μl of sterox was added to the mixture). After 1.5 min, the malachite green reaction was neutralized by the addition of 0.1 ml of sodium citrate solution. The absorbency of the solution was determined at 646 nm. One unit (U) of activity is defined as the amount of enzyme required to produce 1 μmol of PPi (2 μmol Pi) from CTP and NeuAc per minute under the standard assay conditions.

Protein concentrations were determined by using the method described by Lowry et al. [17]. Bovine serum albumin was used as a standard.

2.5. Screening of CMP-NeuAc synthetase positive strain

20 strains of *E. coli* serotype K1 K9 (CGMCC) were grown in 50 ml of medium contained 10 g/l sorbitol, 2.5 g/l yeast extract, 11 g/l K_2HPO_4, 2.5 g/l $(NH_4)_2SO_4$, and 1 g/l $MgSO_4 \cdot 7H_2O$ (pH 6.5) at 37°C overnight. The cells were collected by centrifugation and resuspened in 1 ml of 50 mM Tris–HCl (pH 9.0) containing 1 mM DTT and 20 mM of $MgCl_2$. After sonication, the cell lysate was taken for enzyme assay.

2.6. Gene isolation

Based on the nucleotide sequence encoding for *E. coli* CMP-NeuAc synthetase [11], primers were designed as P1: 5′-CA*GAATTC*ATG-AGAACAAAAATTATTGCG and P2: 5′-CC*GTCGAC*TCATTTAACAATCTCCGCT, and the sites of *Eco*RI and *Sal*I was introduced to 5′ terminal of P1 and P2, respectively. For PCR amplification, genomic DNA from *E. coli* 44277 was digested with *Eco*RI and combined with 100 pmol of each primer in a 100-μl reaction mixture. Thirty cycles (94°C for 1 min, 53°C for 2 min, and 72°C for 1.5 min) were run using Vent DNA polymerase. A 1.3-kb of DNA fragment was amplified and digested with *Eco*RI and *Sal*I, then cloned into the sites between *Eco*RI and *Sal*I of pUC19. The resulting plasmid was designated as pT74. Its construction was finally confirmed by restriction enzyme digestion and DNA sequencing.

2.7. Construction of pET-CS2

CMP-NeuAc synthetase gene cloned in pT74 was mutated by PCR using 5′ primer containing a *Nco*I site (P53: 5′-CT*CCATGG*CTACCAA-GATTATTGCGATAATTCC) and 3′ primer containing *Bam*HI site (P32: 5′-AT*GGATCC*T-TATTTAACAATCTCCGCT). PCR reaction was carried out with Vent DNA polymerase by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. The amplified and digested 1.3-kb *Nco*I/*Bam*HI fragments were inserted into the corresponding sites of pET-15b, and the resulting plasmid was designated as pET-CS2.

2.8. Preparation of modified CMP-NeuAc synthetase

The cell pellet obtained from 1 l of induced culture of *E. coli* DH5α/pET-CS2 (about 6 g) was resuspended in 80 ml of 50 mM Tris–HCl (pH 8.0) and the cells were disrupted by sonication. The cellular debris was removed by centrifugation. The resultant crude extract, with a CMP-NeuAc synthetase specific activity of 0.095 U/mg (99.6 U), was precipitated by the addition of solid ammonium sulfate. The precipitated proteins of 35–60%s were collected and dissolved in 50 ml of 50 mM Tris–HCl (pH 8.0). After dialysis against 3×1 l of 50 mM Tris–HCl buffer (pH 7.6) containing 100 mM NaCl, the enzyme solution was applied to a DEAE-cellulose column (2.5×30 cm) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.6) containing 100 mM NaCl. The column was washed with one column volume of initial buffer to remove unbound proteins. The enzyme was eluted with 2 l of linear gradient of NaCl from 0.1 to 0.25 M. The enzyme fractions were pooled, dialyzed against the initial buffer and

concentrated with Immersible CX-10™ (NMWL 10,000-Da, Millipore). All purification steps were carried out at 4°C.

2.9. Synthesis of CMP-NeuAc

The synthetic reaction was carried out in 10 ml of reaction mixture containing 50 mM Tris–HCl (pH 8.5), 50 mM $MgCl_2$, 0.28 g CTP (0.5 mM), and 0.16 g NeuAc. The reaction was initiated by the addition of 10 U of modified CMP-NeuAc synthetase. The reaction was allowed to proceed for 5 h with stirring at 22°C and the pH was maintained at 8.5 by manual addition of 1 N NaOH. The resultant precipitate was removed by filtration and washed with 25 ml distilled water. Ethanol was added to the filtrate and the mixture (9:1 v/v) was allowed to stand at room temperature for 5 h. The precipitated CMP-NeuAc was isolated by centrifugation (10,000 × g for 10 min) and lyophilized.

The synthesized CMP-NeuAc was analyzed by the thin-layer chromatography (TLC) using 95% ethanol/1 M ammonium acetate (pH 6.0) (7:3) as solvent. The amount of CMP-NeuAc was determined at 254 nm using the commercial CMP-NeuAc as standard.

3. Results and discussion

3.1. Cloning of CMP-NeuAc synthetase gene from E. coli 44277

Among 20 strains of *E. coli* serotype K1K9 obtained from CGMCC, only strain 44277 was found to produce CMP-NeuAc synthetase with a yield of 0.0001 U/ml. Thus, *E. coli* 44277 was used as native source for CMP-NeuAc synthetase gene isolation. A 1.3-kb fragment was amplified by PCR as described in the Experimental (Fig. 1) and cloned into the sites between *Eco*RI and *Sal*I of pUC19 to yield pT74. When the genomic DNA, instead of *Eco*RI digested fragments, was used as template, low amplification and non-specific products were observed, which may be due to the difficulties of denaturing and annealing of long chain of DNA template.

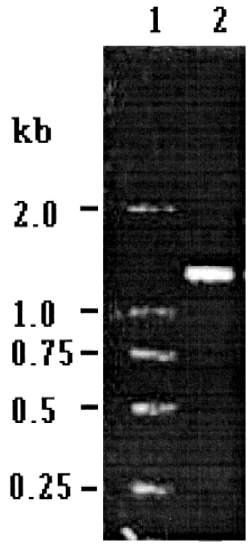

Fig. 1. Electrophoresis (1% agarose) of the primer-directed PCR products from *E. coli* 44277. Lane 1: molecular weight markers; lane 2: the PCR product obtained as described in the Experimental.

The cloned 1.3-kb fragment was confirmed to be CMP-NeuAc synthetase gene by restriction enzyme analysis and sequencing. However, according to the sequencing results, four bases of silent mutation (A153 was substituted by T, A438 by G, A618 by G, and T687 by A) and one codon deletion (Asp230) were found in the coding region (Fig. 2).

3.2. Overexpression of CMP-NeuAc synthetase

Since the extremely low activity of CMP-NeuAc synthetase in wild type *E. coli* is thought to be due to a low efficiency ribosome binding site [18], the Shine–Dalgarno sequence on the expression vector pET-15b was used in this study. In addition, the codon usage should be taken into consideration as another factor affecting the overexpression of CMP-NeuAc synthetase gene in *E. coli* [12]. The native CMP-NeuAc synthetase, therefore, was engineered by

Fig. 2. Nucleotide sequence and deduced amino acid sequence of CMP-NeuAc synthetase from *E. coli* 44277. The nucleotides that differ from the reported sequence are highlighted. The star indicates the deletion of Asp230.

modifying three codons of 5′-terminus and the stop codon. Recently, the occurrence of a low efficient codon for Arg in the translation initial region has been shown to decrease the expression of β-galactosidase [19]. A low-frequency Arg codon was found right behind the initial codon in the synthetase encoding DNA. So the second codon (AGA for Arg2) of the cloned synthetase gene was mutated to GCT encoding for Ala, assuming this amino acid residue, which can occur in all secondary structures of protein would not cause conformational change of active essential N-terminal of the enzyme. With this manipulation, a *Nco*I site containing the initial codon could be introduced for subcloning at the multiple cloning site of pET-15b. Also, to avoid series connection of low-frequency codons in the translation initial region, the codons for Thr3 and Lys4 were mutated to those expected in genes encoding highly expressed *E. coli* proteins [20]. The sequence of engineered 5′-terminus of the enzyme gene is shown in Fig. 3.

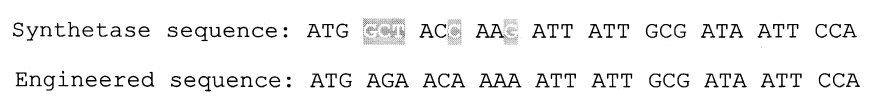

Fig. 3. Sequence of the native and engineered 5′-terminus of the CMP-NeuAc synthetase encoding gene. The mutated nucleotides are marked by highlight.

TGA, a low-frequency stop codon in *E. coli*, was modified to TAA, which was followed by another in-frame TAA on the vector pET-15b and therefore could efficiently terminate the translation. The resulting fragment was then ligated to the *Nco*I/*Bam*HI sites of expression vector pET-15b to yield pET-CS2. This construct, transformed into *E. coli* BL21(DE3)-pLysS, gave expression levels 850-fold higher than that of the wild type and resulted in typical production of 0.085 U/ml of the cell culture. A 49-kDa protein was induced (Fig. 4) and expressed to 5.6–8% of the soluble proteins or 26.5% of the total *E. coli* proteins was achieved. Previously, the overexpression to the extent of 8–10% of the soluble *E. coli* protein was achieved by modifying both the parent Shine–Dalgarno sequence and the codons for the second to the ninth amino acid residue of N-terminal to those expected in genes encoding highly expressed *E. coli* proteins [12]. Only by modifying the ribosome binding site, the overexpression of the modified CMP-NeuAc synthetase at a level of approximately 1000-fold (100 units/l) higher than that of wild type was also achieved. For easy selection of positive clone, the modified enzyme was fused with a decapeptide tag. However, the tagged enzyme was found to be 10 times less stable than the wild type and the difference in pH optimum was observed [13,18]. Our results showed that the modified *E. coli* CMP-NeuAc synthetase, only by replacing six bases in four codons, could be overexpressed at similar levels as compared with that of previously reported.

Besides the expression of the engineered enzyme, Shames et al. also described the expression of native enzyme using vector pKK223 [12]. The plasmid pKKCMP.1 was constructed by inserting a 2.7-kb *Eco*RI/*Hin*dIII fragment from pCMP.3 into pKK223. This construct, which contained the wild type Shine–Dalgarno sequence and the native enzyme encoding DNA, was used for expression of the native enzyme, and the expression of about 1.8% of the soluble *E. coli* protein was obtained. This somewhat low level of expression might be due to the low efficiency of the wild type ribosome binding site. Thus, we tried to express the native synthetase gene by using high efficient ribosome binding site on the expression vector pKK223-3 and pBV220. To carry out these manipulations, the cloned synthetase gene from *E. coli* 44277 was isolated from pT74 and ligated into the sites between *Eco*RI and *Pst*I of vector pKK223-3 behind the tac promoter and pBV220

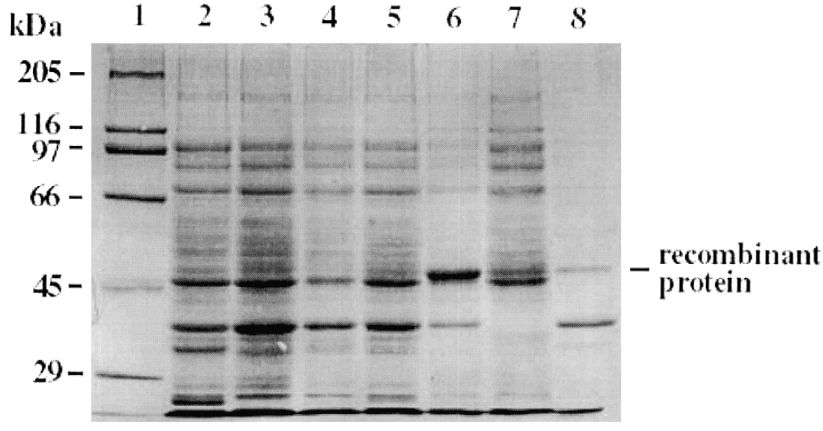

Fig. 4. Analysis of the expression of pET-CS2 in *E. coli* BL21(DE3) by SDS–polyacrylamide gel electrophoresis. A 9.5% polyacrylamide gel stained with Coomassie blue is shown. Lane 1: molecular weight marker; lane 2: host cell lysate; lanes 3 and 4: cell lysate from *E. coli* BL21(DE3) harboring pET-15b without and with induction; lanes 5 and 6: cell lysate from *E. coli* BL21(DE3) harboring pET-CS2 without and with induction; lane 7: induced soluble protein from pET-CS2/*E. coli* BL21(DE3); lane 8: inclusion body from pET-CS2/BL21(DE3) induced with IPTG.

Table 1
Purification of the recombinant CMP-NeuAc synthetase

Step	Protein (mg)	Enzyme activity (U)[a]	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude enzyme	1050	99.6	0.095	1.0	100
$(NH^4)_2SO^4$ fractionation	600	85.4	0.142	1.5	85.7
DEAE-52 chromatography	68	58.9	0.866	9.1	59.1

[a]One unit (U) of activity is defined as the amount of enzyme required to produce 1 μmol of PPi (2 μmol Pi) from CTP and NeuAc per minute at 25°C.

behind the P_L and P_R promoters, respectively. However, no recombinant protein was detected in *E. coli* containing either of these two constructs. These results indicated that, in our case, the cloned gene was not readily expressed by the application of high efficient ribosome binding sites on the expression vectors, although both constructs possessed a ribosome binding site at the optimal distance for expression (10-bp in pKK223-3 and 6-bp in pBV220).

3.3. Preparation of modified CMP-NeuAc synthetase

To increase the engineered CMP-NeuAc synthetase production, the culture conditions of the transformed cells were optimized. The maximum production of the enzyme was obtained by growing the transformed cells in LB medium supplemented with 0.1% glucose (containing 60 μg/ml ampicillin) at 37°C to an OD_{600} of 1.2–1.6 and inducing the expression at 32°C for 4 h using 0.8 mM of isopropyl β-D-thiogalactopyranoside. Under the optimal condition, approximately 100 units of the synthetase were obtained from 1 l of cell culture, while a large portion of the enzyme (about 70%) was found in the insoluble cell pellet. The addition of isopropyl β-D-thiogalactopyranoside at concentrations higher than 0.8 mM caused increased expression of specific 49-kDa protein, however, the enzyme activity in cell extract did not increase. A similar result was also observed when the induction was carried out at 37°C. These results suggest that more insoluble recombinant 49-kDa protein was expressed by the addition of isopropyl β-D-thiogalactopyranoside at concentrations higher than 0.8 mM or by the induction at the optimum temperature. When an exogenous gene is overexpressed in *E. coli*, the folding intermediates would accumulate during the folding of a large quantity of nascent polypeptide. The accumulation of folding intermediate could enhance the interactions of hydrophobic groups on these molecules and cause the formation of insoluble protein. Probably, induction at the temperatures lower than the optimum temperature or by the addition of isopropyl β-D-thiogalactopyranoside at concentrations less than optimum concentration could slow down the synthesis of recombinant protein and therefore decrease the formation of insoluble protein.

The purification of the recombinant enzyme was performed by precipitation with ammonium

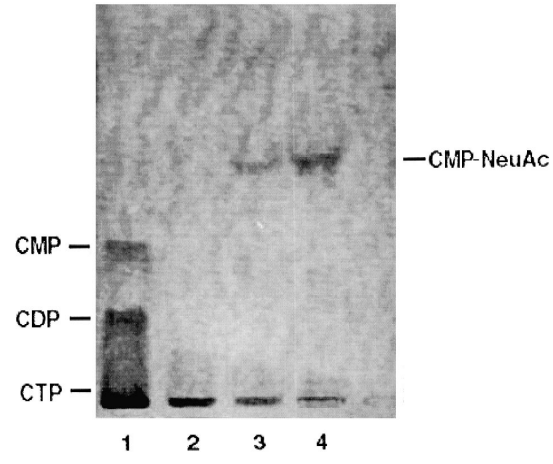

Fig. 5. TLC analysis of the reaction product catalyzed by the racombinant enzyme. The synthetic reaction was carried out as described in the Experimental. The synthesized CMP-NeuAc was precipitated with ethanol, lyophilized and then analyzed by the thin-layer chromatography (TLC) using 95% ethanol/1 M ammonium acetate (pH 6.0) (7:3) as solvent. The amount of CMP-NeuAc was determined as 254 nm. Lane 1: standard CMP and CDP; lane 2: control (reaction mixture without enzyme); lane 3: reaction product; lane 4: CMP-NeuAc standard.

sulfate followed by DEAE-cellulose chromatography. The majority of contaminating proteins was removed in the chromatography. As summarized in Table 1, the enzyme was purified ~9.1-fold from the cell lysate in an overall yield of 59.1%. The specific activity of partially purified enzyme was determined to be 0.866 U/mg, which was about 41% of 2.1 U/mg for the purified CMP-NeuAc synthetase [10]. No phosphatase activity was detected in this enzyme preparation.

3.4. Enzymatic synthesis of CMP-NeuAc

The small-scale synthesis of CMP-NeuAc was carried out in ~90% yield using partially purified recombinant enzyme as catalyst (Fig. 5). The resultant precipitate in the reaction mixture was removed by filtration. The product in the filtrate was purified by precipitation with ethanol and centrifugation. The purity of CMP-NeuAc obtained from the synthetic reaction was judged to be 95%.

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