Overexpression and Purification of Asparagine Synthetase from *Escherichia coli*

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Overexpression of the *asnA* gene from *Escherichia coli* K-12 coding for asparagine synthetase (EC 6.3.1.1) was achieved with a plasmid, pUNAD37, a derivative of pUC18, in *E. coli*. The plasmid was constructed by optimizing a DNA sequence between the promoter and the ribosome binding region. The enzyme, comprising ca. 15% of the total soluble protein in the *E. coli* cell, was readily purified to apparent homogeneity by DEAE-Cellulofine and Blue-Cellulofine column chromatographies. The amino-terminal sequence, amino acid composition, and molecular weight of the purified protein agreed with the predicted values based on the DNA sequence of the gene. Furthermore the native molecular weight measured by gel filtration confirmed that asparagine synthetase exists as a dimer of identical subunits.

L-Asparagine synthetase [L-aspartate: ammonia ligase (AMP-forming) EC 6.3.1.1] catalyzes the synthesis of L-asparagine from L-aspartate and ammonia, while hydrolyzing ATP to AMP and PPI.1) These enzymes are distributed in prokaryotes as *Escherichia coli*2) and *Klebsiella aerogenes*3) and encoded by the *asnA* genes. On the other hand, asparagine synthetases from eukaryotes4–7) use glutamine as the preferred amido nitrogen donor, although they can also use ammonia. These glutamine-dependent enzymes [L-aspartate: L-glutamate amido-ligase (AMP-forming) EC 6.3.5.4] are also found in prokaryotes3,8) and encoded by the *asnB* genes. The steady state kinetic mechanisms of asparagine synthetase have been reported for both the ammonia-dependent and glutamine-dependent enzymes.9–11) In the ammonia-using reaction, the mechanism of the ammonia-dependent enzymes is different from that of the glutamine-dependent enzymes.9,11)

Both types of asparagine synthetase genes have been cloned and sequenced from *E. coli*12,13) and humans.4) The *asnA* gene from *E. coli* codes for a polypeptide of 330 amino acid residues with a molecular weight of 36,700. A high degree of amino acid sequence homology was shown between the glutamine-dependent enzymes (asnB enzyme from *E. coli* and the human enzyme), however, no significant homology was found between the *asnA* and the *asnB* sequences although derived from the same origin, *E. coli*.13) To elucidate these functional differences based on the protein structure, it is essential to obtain a large amount of the purified protein.

We describe the construction of overexpression plasmids of the *asnA* gene from *E. coli* K-12 and a simple method for purifying the enzyme from transformed cells. This is the first time that asparagine synthetase from the *asnA* gene has been purified to apparent homogeneity.

**Materials and Methods**

**Bacterial strains, plages, and plasmids.** Plasmid pMY111 is a pBR322 derivative containing a 2.1-kilobase pair *PstI* fragment of the asparagine synthetase gene (*asnA*) from *Escherichia coli* K-12.2) The overexpression plasmid pKK223-3 was obtained from Pharmacia (Uppsala). Plasmid pUC18 and *E. coli* JM109,14) a host of pUC18 and pKK223-3 derivatives, were purchased from Takara Shuzo Co., Ltd. (Kyoto).

**Restriction enzymes and chemicals.** Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, exonuclease III, mung bean nuclease, and Klenow fragment of DNA polymerase I were purchased from Takara Shuzo Co., Ltd. and Toyobo (Osaka). L-Aspartate was from Wako Pure Chemicals Co., Ltd. ATP, NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were from Oriental Yeast Co. (Tokyo). Adenylate kinase was from Uenichiko Co. (Osaka). DEAE-Cellulofine A-800, Blue-Cellulofine, and Cellulofine GCL-1000S were gifts from Chisso Co. (Mimatoko). All other chemicals used in this experiment were of the purest grades commercially available.

**SDS-polyacrylamide gel electrophoresis.** Protein samples were electrophoresed in 10% polyacrylamide gels in the presence of SDS.15) Gels were then stained with Coomassie Brilliant Blue R-250. To measure the relative amount of asparagine synthetase protein, the stained gels were scanned at 570 nm using a model CS910 chromatoscan (Shimadzu Co.).

**Assay of asparagine synthetase activity.** Protein concentration was measured by the method of Bradford16) with Protein Assay Reagent (Pierce Chemical Co.), and bovine serum albumin as a standard. The protein concentration of the asparagine synthetase in the purified preparation was determined from A418 = 13.9 cm−1 which was calculated from multiplication of the numbers of Tyr and Trp residues by their molar absorption coefficients.17) Asparagine synthetase activity was assayed spectrophotometrically by coupling the formation of AMP to the adenylate kinase, pyruvate kinase, and lactate dehydrogenase reactions. The enzyme solution (50 μl) was added to an assay medium composed of 2 μM adenylate kinase, 10 U pyruvate kinase, 25 U lactate dehydrogenase, 0.24 mM NADH, 1 mM phosphoenolpyruvate, 1.5 mM L-aspartic acid, 20 mM NH₄Cl, 3 mM ATP, 100 mM KCl, 10 mM (CH₃)₂CO₂Mg, 5 mM 2-mercaptoethanol, and 100 mM Tris–HCl buffer (pH 7.8) in a total volume of 1 ml, and the course of the decrease of NADH was monitored at 340 nm. One unit of enzyme was defined as the amount that catalyzed the formation of 1 μmol of asparagine per min at 73°C.

**Plasmid construction and DNA sequencing.** The recombinant DNA techniques were done essentially as described by Maniatis et al.18) Deletion of DNA was carried out by the method of Henikoff et al. with exonuclease III and mung bean nuclease.19) DNA sequences were determined by the dideoxy method.20)

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**Abbreviations:** DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; *asnA*, asparagine synthetase gene from *Escherichia coli* K-12; SD sequence, Shine–Dalgarno sequence; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; LB medium, Luria–Bertani medium; PVDF, polyvinylidene difluoride; lacZ, structural gene of β-galactosidase.
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Amino acid composition and sequence analyses. Amino acid analysis was performed by the method of Spackman et al.\textsuperscript{21} with a Hitachi model 835 amino-acid analyzer. Samples of 2.4 nmole were hydrolyzed in 6 N HCl in vacuo at 110°C for 22, 48, and 72 hr. Cysteine was determined as cysteinic acid after oxidation with performic acid.\textsuperscript{22} The amino-terminal sequence analysis was done with an ABI model 477A gas-phase sequenator. After separation by SDS-polyacrylamide gel electrophoresis, proteins were electrophoretically onto PVDF membrane by the method of Matsudaira.\textsuperscript{23} Then the appropriate band was cut out and sequenced.

Measurement of native molecular weight. The native molecular weight of asparagine synthetase was measured by gel filtration on a Cellulofine GCL-1000 f column (2.6 x 85 cm) equilibrated with 50 mM Tris-HCl buffer containing 0.1 M KCl and 5 mM 2-mercaptoethanol, pH 7.5. The following proteins were used as standards (numbers in parentheses indicate the assumed $M_r$ values): ferritin (450,000), beef liver catalase (240,000), rabbit muscle aldolase (158,000), bovine serum albumin (68,000), hen egg albumin (45,000), chymotrypsinogen A (25,000) and cytochrome $c$ (12,500).

Results and Discussion

Construction of overexpression vector for asparagine synthetase

Expression plasmids for asparagine synthetase were constructed by subcloning $asn\textsuperscript{A}$ gene into a polylinker site of plasmid pKK223-3 or pUC18. Plasmid pKK223-3 has the powerful tac promoter, while pUC18 is a high copy number plasmid with the lac promoter. Both promoters can be induced by adding isopropyl $\beta$-D-thiogalactopyranoside (IPTG).

Plasmid pMY111, containing the wild-type entire $asn\textsuperscript{A}$ gene, was cleaved at the $Nsp(7524)\textsuperscript{I}$ site 43 bases downstream of the Prihnow box of the $asn\textsuperscript{A}$ gene, and treated with Klenow fragment to repair the end of the terminal. Following the $Pst\textsuperscript{I}$ cleavage, a 1.2-kbp fragment containing the $asn\textsuperscript{A}$ gene was isolated from a 0.5% agarose gel and ligated into the $Sma\textsuperscript{I}–Pst\textsuperscript{I}$ site of plasmid pKK223-3. This derivative of pKK223-3 was designated pKNA00. Moreover, the $EcoRI–Pst\textsuperscript{I}$ fragment containing the $asn\textsuperscript{A}$ gene was transferred from the plasmid pKNA00 to the $EcoRI–Pst\textsuperscript{I}$ site of pUC18 to yield plasmid pUNA00 (Fig. 1). Expression of the $asn\textsuperscript{A}$ gene in E. coli JM109 transformed with pKNA00 or pUNA00 was examined after induction of the cells with IPTG. SDS polyacrylamide gel electrophoresis of the cells and the asparagine synthetase activity in the cell-free extracts demonstrated that no detectable amounts of asparagine synthetase were being produced.

In the DNA sequence of the inserted fragment in pKNA00 and pUNA00, there is an inverted repeat between the $Nsp(7524)\textsuperscript{I}$ site and the putative SD sequence (Fig. 1), originating from the wild-type $asn\textsuperscript{A}$ gene. It was considered
that this inverted repeat made a hairpin structure within the mRNA and reduced the efficiency of transcription by acting as a terminator structure or prevented ribosome binding to the mRNA.\textsuperscript{12} To remove this inverted repeat, deletion from the \textit{Nsp} (7524)I site was done. By this method it was also expected that plasmids that have various distances between the SD sequence and the ATG start codon could be obtained. The changes of this distance also could affect the secondary structure of the ribosome binding region within the mRNA. Plasmid pMY111 was cleaved at the \textit{Nsp} (7524)I and the \textit{Dra}I sites, and treated with T4 DNA polymerase to make blunt ends. A 1.5-kbp fragment containing the \textit{asnA} gene was isolated from 1.5% agarose gel and treated with exonuclease III and mung bean nuclease. Following the \textit{Pst}I digestion, the resulting fragments were ligated into the \textit{Smal}–\textit{Pst}I sites of plasmid pKK223-3 (Fig. 1). After these were introduced into \textit{E. coli} JM109, the plasmids expressing the \textit{asnA} gene were selected by SDS polyacrylamide gel electrophoresis of the cellular proteins from these transformants. Four transformants producing a new protein band were obtained. Although this protein appeared to be asparagine synthetase judging from molecular weight, the amount was very little (slightly visible on SDS polyacrylamide gel). Four plasmids were isolated from each transformant and designated as pKNA12, 15, 27, and 37. Next, each \textit{EcoR}I–\textit{Pst}I fragment containing the \textit{asnA} gene was transferred from these pKNA series to the \textit{EcoR}I–\textit{Pst}I site of pUC18 to yield the plasmids pKNA12, 15, 27, and 37. Expression of the \textit{asnA} by the pKNA series was also examined by SDS polyacrylamide gel electrophoresis of the cell extracts prepared from these transformants. In addition to the \textit{EcoR}I–\textit{Pst}I fragments, the \textit{Pst}I fragments were subcloned into M13mp19, and the nucleotide sequence of the region between 5′ and 3′ ends and the translational start codon ATG of the inserted fragment was determined by the dideoxy method (Fig. 2). The protein presumed to be asparagine synthetase was produced in the soluble form at levels ranging from 6.2% to 14.9% of the total cellular protein (Table 1). The highest expression was observed by the use of the plasmid pKNA37 in which the inverted repeat sequence was excised.

\textbf{Purification of asparagine synthetase}

\textit{E. coli} JM109 cells transformed with pKNA37 were grown in 2 liters of LB medium supplemented with 50 \textmu g/ml ampicillin at 37°C with reciprocal shaking. At log phase (about OD\textsubscript{600} = 0.7), IPTG was added to a final concentration of 1 \textmu M. After 14 hr, the cells were harvested by centrifugation at 8000 \(\times\) g for 5 min, and washed in 50 ml of buffer A (20 mm Tris–HCl buffer pH 7.8, 10 mm (\textit{CH}_3\textit{COO})\textsubscript{2}Mg, 1 mm EDTA, 30 mm KCl, and 5 mm 2-mercaptoethanol) and re-collected by centrifugation. The cells were then suspended in 50 ml of buffer A and disrupted by sonication at 0°C. The extract was centrifuged at 25,000 \(\times\) g for 20 min to remove cellular debris. Extraction was repeated after suspending the cellular debris in 50 ml of buffer A.

All procedures below were done at 0–4°C. A cell-free extract was diluted to protein concentration of about 5 mg/ml with buffer B (20 mm Tris–HCl buffer, pH 7.4 containing 10% (w/v) glycerol, 5 mm 2-mercaptoethanol), and applied to a DEAE-Cellulofine A-800 column (5 \(\times\) 18 cm) equilibrated with the same buffer. After washing the column with 0.12 mm KCl in buffer B, elution was done with a linear gradient from 0.12 to 0.4 mm KCl in buffer B. The active fraction was eluted at a KCl concentration of approximately 0.25 mm, and concentrated to about 10 ml on a Amicon UM-10 membrane. The concentrated enzyme was dialyzed against buffer C (the buffer B containing 10 mm (\textit{CH}_3\textit{COO})\textsubscript{2}Mg), and then applied to a Blue-Cellulofine column (2.5 \(\times\) 20 cm) equilibrated with buffer C. The column was washed with 0.1 mm KCl in buffer C, and the enzyme was then eluted with a linear gradient from 0.1 mm KCl, 10 mm (\textit{CH}_3\textit{COO})\textsubscript{2}Mg to 1.0 mm KCl, 0 mm (\textit{CH}_3\textit{COO})\textsubscript{2}Mg in buffer C. The active fraction was concentrated on the Amicon membrane and dialyzed against buffer B. The purified enzyme could be stored at −70°C without loss of activity for at least one month. A typical purification procedure is summarized in Table II and analysis of the enzyme by SDS polyacrylamide gel electrophoresis at various stages in the purification is shown in Fig. 3. The purified enzyme has a specific activity of 22.0 unit/mg which is 49 fold higher than that previously reported by Ceder and Schwartz,\textsuperscript{23} although they assayed the activity by measuring the radioactive asparagine produced. The factor of purification after Blue-Cellulofine was 6.9. It is consistent

![Table 1. Expression Level of the \textit{asnA} Gene in Each Plasmid](image)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Expression level(a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUNA00</td>
<td>ND(b)</td>
</tr>
<tr>
<td>pUNAd12</td>
<td>6.2</td>
</tr>
<tr>
<td>pUNAd15</td>
<td>9.6</td>
</tr>
<tr>
<td>pUNAd27</td>
<td>7.6</td>
</tr>
<tr>
<td>pUNAd37</td>
<td>14.9</td>
</tr>
</tbody>
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\* Relative amount of asparagine synthetase protein to the total cell protein measured from the intensities of protein bands.

\(b\) Not detectable.
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| Table II. Purification of Asparagine Synthetase Produced by *E. coli* JM109/pUNAd37 |
|---------------------------------|-----------------|-----------------|---------------|-------------|
|                                | Total protein   | Specific activity | Total activity | Recovery | Purity |
| Crude extract                  | (mg)            | (unit/mg)         | (unit*)        | (%)       | (-fold) |
| DEAE-Cellulose A-800           | 200             | 14.1             | 2811           | 94.9      | 4.4     |
| Blue-Cellulose                 | 68              | 22.0             | 1496           | 50.6      | 6.9     |

* One unit of enzyme was defined as the amount producing 1 μmol of Asn per min under the standard assay conditions.

![SDS-Polyacrylamide Gel Analysis of Enzyme Purification](image)

Fig. 3. SDS-Polyacrylamide Gel Analysis of Enzyme Purification. Lane 1, cell-free extract of *E. coli* JM109 not transformed; lane 2, cell-free extract of JM109 transformed with pUNAd37; lane 3, DEAE-Cellulose A-800 fraction; lane 4, Blue-Cellulose fraction.

with the fact that the asparagine synthetase protein is about 14% of the total cellular protein based on the intensities of protein bands.

**Protein characterization**

The N-terminal amino acid sequence of the asparagine synthetase produced by *E. coli* JM109/pUNAd37 was analyzed by a gas-phase sequenator. The first 5 residues were Met-Lys-Thr-Ala-Tyr. This sequence agreed exactly with that predicted from the DNA sequence of the *asnA* gene. An amino acid composition analysis of the purified enzyme was also done, and the results are compared with those expected from the DNA sequence (Table III). The disagreement is within the usual limit of variation, at most 5%. Furthermore, the native molecular weight was measured by gel filtration as 71,600. This also agreed with the fact that *E. coli* asparagine synthetase exists as a dimer of identical subunits.12)

In conclusion, we have constructed an overexpression plasmid for *E. coli* asparagine synthetase that produces the enzyme in the form expected. This has enabled us to prepare a large amount of the enzyme by a simple purification procedure. This work can now form the basis of a study of the enzyme by X-ray crystallography and protein engineering.

**Acknowledgments.** We are indebted to Dr. M. Takanami for providing us the original *E. coli* strain carrying plasmid pMY111.

**References**