0273-2289/01/95/0093/$12.25

Cloning and Expression of L-Asparaginase Gene in *Escherichia coli*

Yingda Wang, Shijun Qian,*
Guangzhen Meng, and Shuzheng Zhang

Department of Enzymology, Institute of Microbiology, Academia Sinica, Beijing 100080, China, E-mail: Qiansj@sun.im.ac.cn

Received June 25, 1998; Accepted June 9, 1999

Abstract

The L-asparaginase (ASN) from *Escherichia coli* AS1.357 was cloned as a DNA fragment generated using polymerase chain reaction technology and primers derived from conserved regions of published ASN gene sequences. Recombinant plasmid pASN containing ASN gene and expression vector pBV220 was transformed in different *E. coli* host strains. The activity and expression level of ASN in the engineering strains could reach 228 IU/mL of culture fluid and about 50% of the total soluble cell protein respectively, more than 40-fold the enzyme activity of the wild strain. The recombinant plasmid in *E. coli* AS1.357 remained stable after 72 h of cultivation and 5 h of heat induction without selective pressure. The ASN gene of *E. coli* AS1.357 was sequenced and had high homology compared to the reported data.

Index Entries: *Escherichia coli*; L-asparaginase; gene expression; plasmid stability; DNA sequence.

Introduction

The enzyme L-asparaginase (ASN) (EC.3.5.1.1; L-asparagine amino-hydrolase) *(1)* catalyzes the deamination of L-asparagine (Asn) to L-aspartate and ammonia. The enzyme has been isolated from a variety of sources: animals and plant cells, yeast, fungi, and bacteria. It is identified as an effective agent in the therapy of certain types of lymphoma and leukemia. The tumor cells rely on an exogenous supply of Asn for their proliferation, and this enzyme causes selective death of asparagine-dependent tumor cells by depriving them of asparagine. ASN can induce complete remission in up to 80% of patients suffering from acute lymphoblastic leukemia. The principal sources of asparaginase used extensively are from the bacteria

*Author to whom all correspondence and reprint requests should be addressed.

Escherichia coli or *Erwinia chrysanthemi (2)*. As a result, they have been the subject of intensive study.

The *E. coli* L-ASN, a periplasmic enzyme, is a tetramer with a subunit molecular mass of 3.2×10^4, and p*I* is 4.8 *(3)*. The amino acid and the nucleotide sequences of the *E. coli* encoding ASN have been determined *(4,5)*. The ASN gene of *E. coli* K-12 has been shown to encode a 22-amino acid signal peptide at its N-terminus for a periplasmic protein.

The enzyme is produced commercially as a therapeutic product for certain types of leukemia and disseminated cancer. However, even the best strains currently available, namely *E. coli* AS1.357 and *E. chrysamthemi* NCPB1066, yield quantities of ASN that are lower than desirable. Therefore, one of the challenges of the large dosage is the cost of the final product, which means that a cost-effective production process is vital.

To scale up production of ASN, we tried to construct a recombinant plasmid containing ASN gene from *E. coli* AS1.357 *(6,7)*, which was screened by our laboratory and has been used in industry, to express at high levels in some kinds of *E. coli* strains, and to sequence the ASN gene. We also studied the stability of the recombinant plasmid PASN in host strains.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

E. coli AS1.357 was screened by our laboratory; *E. coli* JM105, JM109, DH5α, and TG1 were used as the expression host; and the DH5α was used as the cloning host for DNA sequencing. The plasmids pBV220 *(8)* and pUC19 were used for expression and cloning, respectively; *E. coli* strains were cultured in Luria broth (LB) (1% tryptone [Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK], 0.5% yeast extract [Oxoid], 1% NaCl); and ampicillin was added to media at a final concentration of 100 μg/mL for the selection and growth of transformant.

Chemicals

The restriction enzymes and T4 DNA ligase were from Sino-American Biotechnology. The polymerase chain reaction (PCR) kit was from Sagon Biotechnology (Shanghai, China). The sequencing kit was from Perkin Elmer. ASN was from Tianjin Biochemical Pharmaceutical.

DNA Isolation

E. coli DNA

Midlog cells of *E. coli* were harvested and DNA was extracted as described by Keller and Manak *(9)*.

Plasmid DNA

Plasmid DNA was isolated and purified as described by Sambrook et al. *(10)*. The rapid boiling method *(11)* was employed for small-scale plasmid isolation for screening purposes.

PCR and Electrophoresis Techniques

PCR and electrophoresis of DNA fragments were carried out according to standard methods *(10)*. Two oligo primers designed for PCR amplification were based on the nucleic acid sequence of the reported *E. coli* K-12 (no. M34234). The cloning sites of *Eco*RI and *Sal*I were incorporated into the N- and C-terminal primer sequence of the ASN, respectively. The primer sequences are as follows:

Primer-1: 5'-TAGAATTCATGGAGTTTTTCAAGAAGACG-3'

Primer-2: 5'-AAGTCGACATTAGTACTGATTGAAGATC-3'

Thirty-six cycles of PCR were performed with the following temperature profile in 25 µL: denaturation at 94°C for 45 s, annealing at 60°C for 1 min, extension at 72°C for 1.5 min except for the final cycle in which the extension proceeded for 10 min.

The product was electrophoresed in agarose gels (0.8%) using TAE buffer *(10)*. PBR322/*Bst*NI were used as molecular weight standards. A 1-kb or so amplified gene product was recovered from low melting temperature agarose gels.

General Recombinant DNA Procedures

Restriction endonucleases and T4 DNA ligase were used according to the manufacturer's instructions and transformation of *E. coli* was as described by Sambrook et al. *(10)*.

The PCR product and the pBV220 DNA were digested with *Eco*RI and *Sal*I, respectively. Then both digested fragments were purified from low melting temperature agarose gels and ligated. The resulting recombinant plasmid was transformed to *E. coli* host strains.

Gene Expression and Enzyme Assay

Cells were grown at 30°C in LB with ampicillin (100 µg/mL) to an A_{600} of about 0.3, then at 42°C for 4 h. ASN activity was determined by the method of Peterson and Ciegler *(12)*. Cell-free extracts of *E. coli* strains were prepared by sonication and removal of cell debris by centrifugation. The protein samples were electrophoresed on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Sambrook et al. *(10)*. The level of expression was the percentage of asparaginase protein in the total soluble cell protein and was determined by densitometric scanning of products on SDS-PAGE.

DNA Sequencing of the ASN Gene

ASN gene fragment was subcloned into pUC19 vector, purified by the Wizard® DNA purification System (Promega), and sequenced by the dideoxy mediated chain-termination method *(10)*. The entire sequence was

determined from both strands with ABI PRISMTM377 DNA sequencer. The two sequencing primers were as follows:

F primer (M13-47): CGCCAGGGTTTTCCCAGTCACGAC
R primer (RV-M): GAGCGGATAACAATTTCACACAGG

Stability of pASN in E. coli

Monitoring of plasmid stability was carried out as described previously with some modification *(13,14)*. Bacteria were grown overnight on LB with ampicillin (100 µg/mL), and the culture fluid was diluted 1:10,000 and transferred to 50 mL of LB medium without ampicillin at 30°C with shaking at 200 rpm. Cultures were renewed every 12 h using fresh LB medium, and serial batch cultivation was carried out in shake flasks for 48 h *(13)*. When the last culture gave an absorbance of about 0.3×10 at 600 nm, heat induction was carried out. At the indicated time, a portion of the culture was plated on LB plates, and 100 resulting colonies were placed on LB ampicillin plates. Ampicillin-resistant colonies were also tested for the structural stability of the ASN gene by examining the enzyme activity of the colonies.

Results

Construction of Recombinant Plasmid pASN

By the method of PCR described previously, one major band of approx 1000 bp, the expected size of the authentic ASN gene, was generated on 0.8% agarose gel electrophoresis of the products.

The purified fragments from agarose gel were digested with *Eco*RI and *Sal*I. The *Eco*RI-*Sal*I fragment was directly cloned into the appropriate sites of pBV220 to give the plasmid pASN (Fig. 1). The recombinant plasmid was digested with *Eco*RI and *Sal*I to generate two fragments (Fig. 2), corresponding to the length of ASN gene and pBV220.

Expression of the L-Asparaginase Gene in E. coli

We transformed *E. coli* JM105, TG1, JM109, DH5α, and AS1.357 with pASN. Cells were grown at 30°C in LB with ampicillin (100 µg/mL) for 5.5 h, then at 42°C for 4 h, and we determined the ASN activity in resultant recombinant clones to obtain the higher levels of expression of ASN. Table 1 shows the highest value of expression in different strains. The enzymatic activity and expression level of *E. coli* harboring pASN were 75.1–167.6 IU/mg of dry cell mass and approx 50%, respectively. It was surprising to find the *E. coli* AS1.357 harboring pASN product at higher levels of ASN in shorter culture times.

The subunit molecular weight of the enzyme was 36,000 Daltons by SDS-PAGE (Fig. 3), which is the same value as the ASN from *E. coli* AS1.357. In both cases, the enzymes appeared to be tetramer as determined by electrophoresis (data not shown).

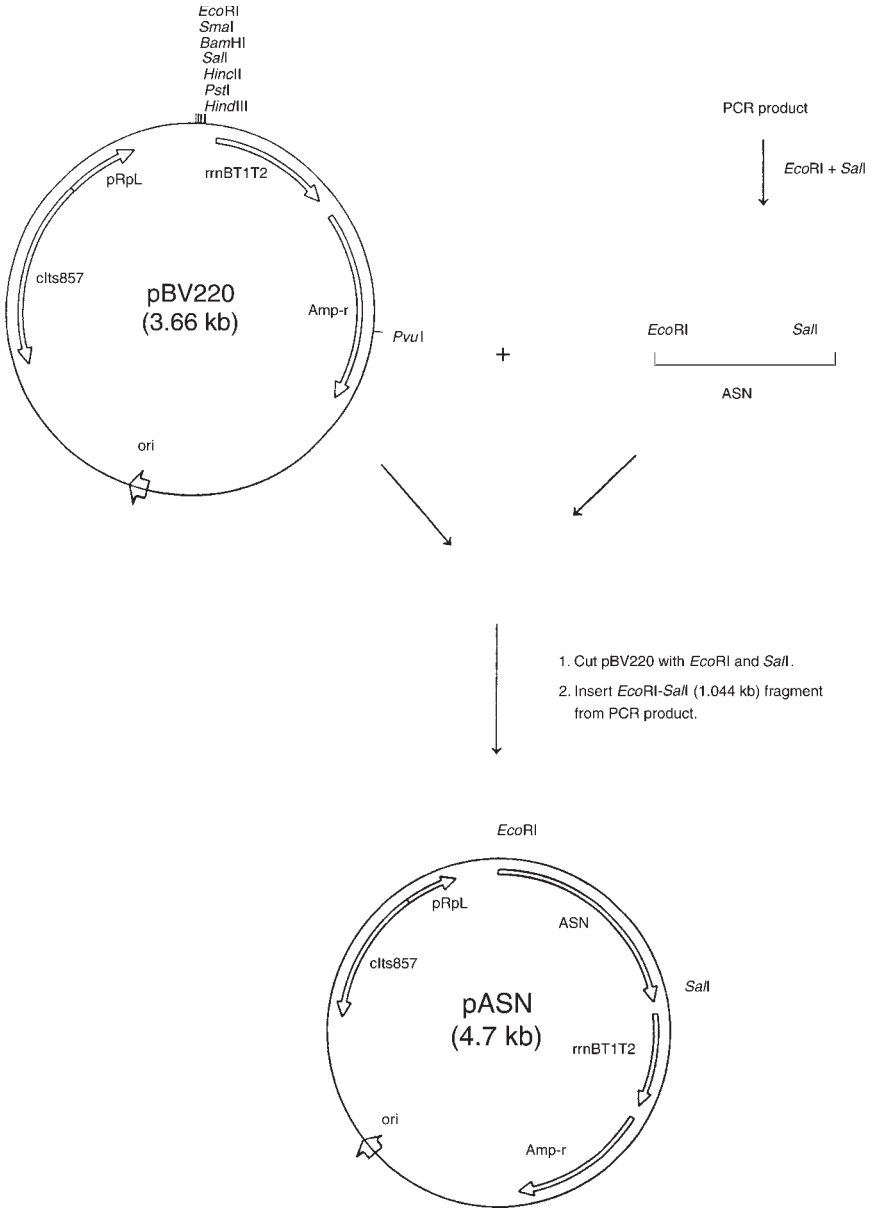

Fig. 1. Construction of recombinant plasmid pASN.

Sequencing of the L-Asparaginase Gene

The complete sequence of 1044 bp given in the sequencing result includes the open reading frames of L-asparaginase gene, from an ATG to a stop codon. The first 22 amino acids preceding the mature N-terminus encode a typical cleaved signal peptide and closely resemble sequences identified as the N-terminus of other secreted protein *(14)*. The genes of

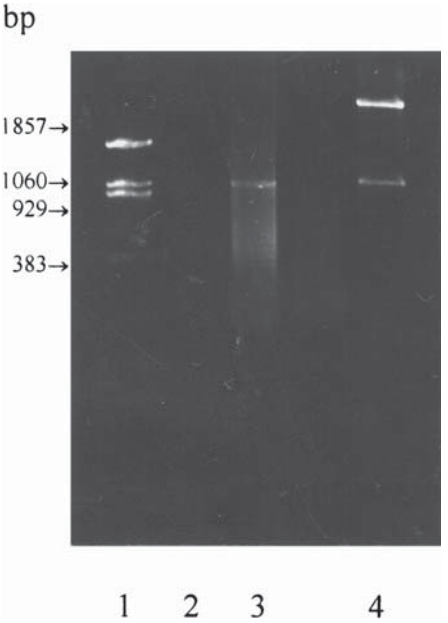

Fig. 2. Agarose gel electrophoresis of pASN digested with restriction enzymes. Lane 1, pBR322/*Bst*NI; Lane 2, pBV220/*Eco*RI + *Sal*I; Lane 3, PCR amplification product of L-ASN gene; Lane 4, pASN/*Eco*RI + *Sal*I.

Table 1
Expression of L-ASN Gene
from Recombinant Plasmids pASN in Different Host Strains

Host strain	JM105	JM109	TG1	DH5α	AS1.357
Enzyme activity (IU/mL)	153.00	58.60	167.00	42.30	228.00
Dry cell mass/mL culture (mg/mL)	1.32	0.78	1.27	0.38	1.36
Enzyme activity/ mg dry cell mass (IU/mg)	115.90	75.10	131.50	110.50	167.60
Level of expression (%)	57.90	74.80	49.40	46.90	43.10

E. coli and K-12 have a high degree of primary homology: both have 1044 bp, and only 33 bp are different.

Stability of pASN in E. coli *TG1 and AS1.357*

We tested the stability of pASN in TG1 and AS1.357 for up to 50 generations without antibiotic selection. Figure 4 shows that the recombinant plasmid pASN in *E. coli* AS1.357 was almost 100% stable without selective pressure, and also was quite stable in *E. coli* TG1 after 48 h of culture and 5 h of heat induction. No structural instability was observed in any of the hosts (data not shown).

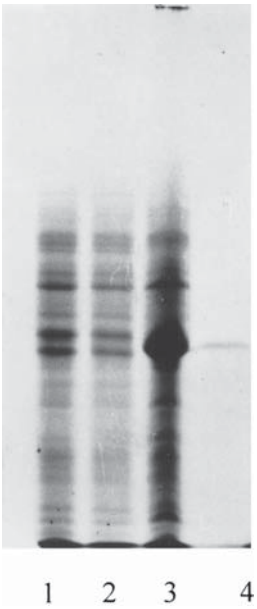

Fig. 3. SDS-PAGE analysis of pASN expression product. Lane 1, AS1.357; Lane 2, AS1357 (pASN) cultured at 30°C; Lane 3, AS1.357 (pASN) expression product; Lane 4, L-asparaginase produced by Tianjin Biochemistry Pharmaceutical Factory.

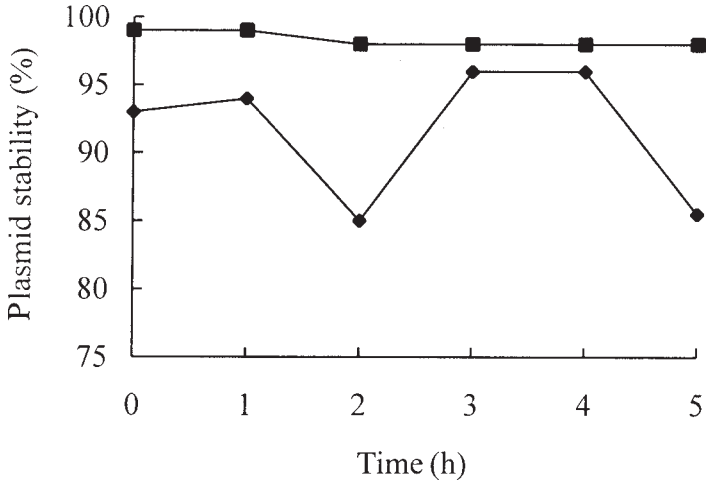

Fig. 4. Stability of pASN (■) and (◆), plasmid stabilities in *E. coli* AS1.357 and TG1, respectively.

Discussion

It can be seen from the procedures and results we have described that the ASN gene coding for ASN has successfully been cloned and expressed at a high level in several *E. coli* host strains. The ASN gene amplified by PCR

was cloned into pBV220 to give the recombinant plasmid pASN. The high level of expression was about 50% soluble cell protein, and the enzyme activity was 75.1–167.6 IU/mg of dry cell mass.

ASN production by *E. coli* and *E. chrysanthemi* has been studied previously. However, the research on molecular cloning and gene expression of ASN gene has scarcely been reported. Gilbert et al. *(15)* and Liu et al. *(16)* have cloned and expressed asnB gene in *E. coli* and *E. carotovora*, respectively. The enzyme activities of their engineering strain were 49 and 106 IU/mL, respectively. The promoters of expression vector used were *lac* and *tac*, the chemical induction promoter, so the expression of recombinant plasmids needs the induction of IPTG, which is costly and inconvenient in large-scale fermentations. In this study, we used the expression vector with pRpL promoter, the heat induction promoter, the expression of the recombinants is advantageous to the chemical inducer in large-scale fermentations. The engineering strain produced up to 40-fold higher activity as compared to the wild-type *E. coli* AS1.357 (4.6 IU/mL); hence, the engineering strain is a suitable strain to produce ASN.

Of all the host strains tested, DH5α, which was reported to be an effective host strain *(8,14)*, is far from ideal. The *E. coli* AS1.357 harboring pASN produced a higher level of the enzyme. This finding suggests that the host genetic background can play an important role in the final yield of accumulated gene product and that this strain holds the appropriate traits for regulation of gene expression *(15)*. As the industrial microorganism previously used, we think the genetic background of *E. coli* AS1.357 is more suitable for high-productivity yields of the recombinant plasmid.

In many cases, strains carrying recombinant high-level expression systems have even been found to be unstable, because usually a high metabolic load tends to be overgrown and affects the plasmid segregation *(17)*. Thus, we determined the stability of pASN in *E. coli* AS1.357 (Fig. 4). Our results show that pASN is stable in two strains and more stable in *E. coli* AS1.357. This may suggest that the stability of pASN depends on the genetic background of the strain.

In the amino acid sequence derived from the nucleotide sequence of *E. coli* AS1.357, two amino acids—Ala_{27} and Asn_{263}—differ with the sequence of K-12—Val_{27} and Thr_{263}—but differ even greater with the amino acid sequence reported by Maita et al. *(5)*. These differences may represent differences in strain.

References

1. Schwartz, J. H., Reeves, J. Y., and Broome, J. D. (1966), *Proc. Natl. Acad. Sci. USA* **56,** 1516–1519.
2. Nowak-Goettl, U., Werber, G., Ahlke, E., and Juergens, H. (1997), *Haematol. Blood Transfus.* **38(Acute leukemias VI),** 497–501.
3. Epp, O., et al. (1971), *Eur. J. Biochem.* **20,** 432–437.
4. Bonthron, D. T. (1990), *Gene* **91,** 101–105.
5. Maita, T., Morokuma, K., and Matsuda, G. (1974), *J. Biochem.* **76,** 1351–1354.

6. Qiu, X. B., Hao, F. X., Qian, S. J., Chen, J. Y., and Meng, G. Z. (1973), *Acta Microbiologica Sinica* **13(1),** 59–62.
7. Meng, G. Z., Hao, F. X., Qian, S. J., and He, Z. X. (1985), in *Molecular Architecture of Proteins and Enzymes*, Bradshaw, R. A. and Tang, J., eds., Academic, New York, pp. 135–154.
8. Zhang, Z. Q., Hong, L. L., and Hou, Y. D. (1990), *Chinese J. Virol.* **6(2),** 111–116.
9. Keller, G. H. and Manak, M. M. (1989), in *DNA Probes*, Stockton Press, NY, pp. 20–24.
10. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), in *Molecular Cloning: A Laboratory Manual*, Nolan, C., ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 1.25–1.41.
11. Berghammer, H. and Auer, B. (1993), *Biotechniques* **14(4),** 524–528.
12. Peterson, R. E. and Ciegler, A. (1969), *Appl. Microbiol.* **17(6),** 929,930.
13. Vehmaanpera, J. O. and Korhola, M. P. (1986), *Appl. Microbiol. Biotechnol.* **23,** 456–461.
14. Chen, W., He, B. W., Zhang, J. H., Zhou, J., and Chen, N. Y. (1997), *Acta Microbiologica Sinica* **37(4),** 270–275.
15. Gilbert, H. J., Blazek, R., Bullman, H. M. S., and Minton, N. P. (1986), *J. Gen. Microbiol.* **132,** 151–160.
16. Liu, J. J., Li, J., Wu, W. T., and Hu, M. Q. (1996), *J. China Pharm. Univ.* **27(11),** 696–700.
17. Pines, O. and Inouye, M. (1996), in *Methods in Molecular Biology*, vol. 62, *Recombinant Gene Expression Protocols*, Tuan, R., ed., Humana, Totowa, NJ.