Cloning, Expression and Purification of Arylsulfate Sulfotransferase from *Eubacterium* A-44

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A gene (*astA*) encoding arylsulfate sulfotransferase (ASST), which transfers a sulfate group from phenolic sulfate esters to phenolic acceptors, was cloned from a *Eubacterium* A-44 genomic library. The probe (1.5 kb fragment) for the *astA* gene was prepared from the PCR product of the primers produced using two internal amino acid sequences of ASST, which had been purified from *Eubacterium* A-44. The *astA* gene was cloned into the pKF3vector. Its sequence revealed a 1863 bp open reading frame (ORF) encoding a protein containing 620 amino acids with a secretory signal peptide, and showed 91% homology (identity) to *Eubacterium rectale* IIIH previously reported. The cloned *astA* gene was expressed under the T7 promoter of the expression vectors, pET-39b(+) and pET-26b(+), in *Escherichia coli* BL21 (DE3), and the expressed ASSTs were purified using His Bind column chromatography. The specific activities of the purified ASSTs were 25.6 $\mu\text{mol}/\text{min}/\text{mg}$ and 37.1 $\mu\text{mol}/\text{min}/\text{mg}$, respectively.

Key words arylsulfate sulfotransferase; *Eubacterium* A-44; *astA* gene; gene cloning

Arylsulfate sulfotransferase (ASST) was purified from *Eubacterium* A-44,^{1,2)} which is an anaerobe from the human intestinal microflora that metabolizes picosulfate sodium.³⁾ ASST catalyzes the transfer of a sulfate group from various phenyl sulfate esters to phenolic acceptor substrates. Although Goldberg *et al.* cloned the ASST gene (*astA*) from *Eubacterium rectale* IIIH, the physiological action of ASST is not completely understood.⁴⁾ Nevertheless, the enzyme has potential use in the *in vitro* O-sulfation of peptide hormones that require the sulfoconjugation of a hydroxyl group from tyrosine for their biological activity, such as cholecystokinin and hirudin.⁵⁾

In this study, the *astA* gene encoding ASST of *Eubacterium* A-44 was cloned and compared with the previously reported recombinant ASST in order to determine its use industrially. The cloned *astA* was expressed in *Escherichia coli*, and its substrate specificity from the purified recombinant clones was determined.

MATERIALS AND METHODS

Construction of *Eubacterium* A-44 Genomic Library

The following sequence was used to clone the *astA* gene of *Eubacterium* A-44. First, ASST was purified according to previously reported methods,¹⁾ and two internal amino acid peptides, AST 16-1 (5'-ACRGCRGARACRGGRTARCA-3') and AST 16-2 (5'-CGRTARTARTTNCCNGGNACRTG-3') that were obtained by trypsin digestion were sequenced. The two primers were synthesized according to their sequences and PCR was performed. The PCR product was labeled with DIG and used as a hybridization probe. In order to prepare the genomic library of *Eubacterium* A-44, the chromosomal DNA was partially digested with HindIII at 37 °C for 2 h and size-fractionated on a 0.5% TAE agarose gel. A portion of the digested chromosomal fragments (2–3 kb) was removed from the gel. The DNA was extracted and ligated to the pKF3 vector (Takara, U.S.A.), which was digested with the same enzyme, and transformed into *E. coli* TH2 competent cells (Takara, U.S.A.). The positive clones were screened.

Southern blotting and DNA sequencing were used to confirm the cloned ASST gene.

Expression and purification of *Eubacterium* A-44 ASST

In order to express the *astA* gene, four primers for the full length of ASST, which contain a signal peptide or/and stop codon, were synthesized and amplified by PCR. Two 5' primers (BM-A44-F1 and BM-A44-F2) contained a common unique BamHI site: BM-A44-F1 (5'-GAGAGAGGATCC-NATGTCAGTAAAATA-3') has a signal peptide but BM-A44-F2 (5'-GAGAGAGGATCCNGAAGCAGAGCA-3') does not. Two 3' primers (BM-A44-R1 and BM-A44-R2) have a common unique HindIII site: BM-A44-R1 (5'-GAGAGAAAGCTTGACGTGATCGTA-3') does not have a stop codon whereas BM-A44-R2 (5'-GAGAGAAAGCTTTTAGCAGCTGATCGTA-3') does. Each PCR product, which contained a BamHI site at the 5' ends and a HindIII site at the 3' ends, was subcloned into the pGEM-T easy vector (Promega, U.S.A.), and transformed into the *E. coli* JM109 competent cells (Promega, U.S.A.). The recombinant plasmid was isolated using a plasmid SV kit (Generalbio, Korea), purified from 0.8% agarose gel and ligated into the pET-39b(+) and pET-26b(+) expression vectors (Novagen, U.S.A.), which contained the inducible T7 promoter. Both pET-39b(+) and pET-26b(+) were used for the purpose of enhancing the solubility. The pET-39b(+) vector contains DsbA, which is the periplasmic enzyme that catalyzes the formation and isomerization of the disulfide bond. The pET-26b(+) vector contains an N-terminal pelB signal sequence for potential periplasmic localization. The PCR products were ligated into the pET-39b(+) vector containing the 6 His tag backward or forward using the primer with the stop codon, and in the pET-26b(+) vector with the 6 His tag only backward using the stop codon-excluded primer (Fig. 1). The ligated plasmids were transformed into the expression host, *E. coli* BL21 (DE3), which was confirmed by restriction enzyme analysis.

The ASST expressed in *astA*-cloned *E. coli* was purified by culturing the bacteria to the mid-log phase (OD₆₀₀ 0.6–1) in 100 ml of LB broth containing 30 $\mu\text{g}/\text{ml}$ of kanamycin.

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Fig. 1. Ligation Maps of the Arylsulfate Sulfotransferase Gene and Expression Vectors

(1) and (2) were ligated in the pET 39b(+) vector. (3) and (4) were ligated in the pET 26b(+) vector. (2) and (4) contained the vector signal peptide (without the signal peptide of *astA*). The pET vector contains the backward 6 His-tag(tag) and the 39b(+) vector contains the forward 6 His tag. SP means a signal peptide, SC indicates a stop codon and the tag is 6 His-tag.

The cells were then induced with 1 mM IPTG at 20 °C for 16 h. The cultured cells were centrifuged at 10000×*g* for 10 min at 4 °C, resuspended in 10 ml of an ice-cold 1×binding buffer [20 mM Tris-HCl (pH 7.9) containing 500 mM NaCl and 5 mM imidazole, Novagen, U.S.A.], sonicated at 4 °C and centrifuged at 14000×*g* for 20 min. The resulting supernatant was applied to the His Bind column (Novagen, U.S.A.), washed stepwise with a 1×binding and 1×wash buffer [20 mM Tris-HCl (pH 7.9) containing 500 mM NaCl and 60 mM imidazole, Novagen, U.S.A.] and eluted with a 1×elution buffer [20 mM Tris-HCl (pH 7.9) containing 0.5 M NaCl and 1 M imidazole, Novagen, U.S.A.].

The ASST expressed in the pET-39b(+) vector was dialyzed the eluate against the 20 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl and 2 mM CaCl₂. The dialyzed solution was incubated with the recombinant enterokinase (Novagen, U.S.A.) at 20 °C for 3 h. The cleaved protein mixture was then added to the His Bind Column, and eluted with a 1×binding buffer. SDS-PAGE analysis confirmed that the ASST of the eluted fractions was a homogenous protein with the fusion segment (DsbA) completely removed.

Determination of Protein The quantity of the protein was determined using the Bradford method.⁶⁾

Assay of ASST Activity The reaction mixture (total volume of 0.63 ml) used for the ASST activity assay contained 30 μl of 50 mM *p*-nitrophenyl sulfate (PNS), 0.29 ml of 20 mM tyramine (occasionally other acceptors at the indicated concentration), 0.21 ml of 0.1 M Tris-HCl buffer, pH 8.0, and 0.1 ml of the enzyme solution. The reaction mixture was incubated at 37 °C for 5 min, and quenched by adding 1 N NaOH (0.4 ml). The absorbance at 405 nm was measured.⁶⁾

Fig. 2. Nucleotide Sequence of the *Eubacterium* A-44 *astA* Gene Regulation Region

The 5'-ends of the *Eubacterium* A-44 ASST mRNA (transcriptional start site of ASST gene) is indicated in boldface (A) with the dextral arrow (TS) in the DNA sequence. The proposed Shine-Dalgarno (SD) sequence and the consensus sequence for the sigma 70-type promoter are underlined.

RESULTS AND DISCUSSION

The *Eubacterium* A-44 chromosomal DNA was partially digested with HindIII, ligated to the HindIII arms of the pKF3 vector and transformed *E. coli* TH2 competent cells. Two positive colonies were obtained using the DIG-detection method with the probe (1.5 kb) being prepared by PCR of the two primers synthesized according to the internal amino acid sequences (the sequences were H-V-P-G-N-Y-Y-R and T-A-E-T-G-Y-H). The colonies were identified from the libraries (the 75th recombinant plasmid). The inserted DNA (about 2 kb), which was identified from the positive clones, was similar in size to the fragment previously prepared from the HindIII digested DNA. The inserted DNA was sequenced and analyzed using the GenBank homology search Blast system, and the ORF finder program in the NCBI and genecutter program. The *Eubacterium* A-44 *astA* gene consists of a 1863 bp sequence encoding a protein with 620 amino acid residues containing a signal peptide (16 amino acids). The putative ribosome-binding site (Shine-Dalgarno sequence, GGAGGT) was located 7 bp upstream from the start codon (ATG).⁷⁾ A sequence of the transcriptional start region of the A-44 *astA* gene was located 65 nucleotides upstream from the translational start codon (AUG) of ASST, and the putative sigma 70 type promoter sequences of both TTTACA (resembling -35 sequence) and GGTATTAT (resembling -10 sequence) were located at positions -95 and -77, respectively.⁸⁾ The three sequences related to *Eubacterium* A-44 were obtained from database searches of GenBank using BLAST2. The sequence of the A-44 *astA* gene was compared with the ASSTs from *Eubacterium rectale* IIIH, *Klebsiella* K-36⁹⁾ and *Enterobacter* AR-37.¹⁰⁾ The gene of *Eubacterium rectale* showed 91% homology to the *Eubacterium* A-44 but the other *astA* genes did not. The amino acid sequence of the A-44 *astA* amino acid was compared with the ASSTs from *Eubacterium rectale* IIIH, *Klebsiella* K-36⁹⁾ and *Enterobacter* AR-37.¹⁰⁾ These three amino acids showed 96%, 23% and 22% (positives 98%, 38%, 39%) homology (identity) to the *Eubacterium* A-44, respectively. (Fig. 3). The nucleotide sequence of the *Eubacterium* A-44 *astA* gene was deposited in GenBank (accession number, DQ683024).

In order to investigate the industrial applications of the recombinant *astA* gene in *E. coli*, *astA* was expressed by inserting the cloned gene with and without the codon of its signal peptide into the BamHI and HindIII sites of pET-39b(+) and pET-26b(+) vector, which has a 6 His-tag. The inserted vectors were then transformed into the expression host *E. coli* BL21(DE3), cultured, and induced with 1 mM IPTG at 20 °C for 16 h. The cultured cells were collected by centrifugation.

The asterisks below the aligned sequences indicate the same amino acids in all four sequences. The positions with colons below contain a strongly conserved residue, and the periods indicate more weakly conserved residues. The gaps introduced to align the protein sequences optimally are indicated by dashes.

gation and their ASST activity was measured (Table 1). The proteins were well expressed by the *astA* gene with the codon of its signal peptide and ASST activity that was independent of the DsbA was observed. On the other hand, although the *astA* gene without the codon of its signal peptide was expressed, no ASST activity was observed. These results

Strain	Activity (U/mg)
<i>Eubacterium</i> A-44	0.1
<i>E. coli</i> BL21(DE3)-pET-39b(+) with astA signal peptide	1.4
<i>E. coli</i> BL21(DE3)-pET-39b(+) without astA signal peptide	0
<i>E. coli</i> BL21(DE3)-pET-26b(+) with astA signal peptide	2.5
<i>E. coli</i> BL21(DE3)-pET-26b(+) without astA signal peptide	0

(A) ASST expressed in the astA-cloned *E. coli* BL21(DE3). The astA gene with (3) or without the signal peptide (5) inserted in the pET-39b(+) or with (7) or without the signal peptide (9) in the pET-26b(+) was transformed into the *E. coli* BL21(DE3), induced with 1 mM IPTG at 20 °C for 16 h, collected at 5000×g for 20 min and examined by SDS-PAGE. (2), (4), (6) and (8) were carried out using the same methods reported for (3), (5), (7) and (9) without the IPTG treatment, respectively. (1) and (10) indicate the molecular markers. (B) ASST was purified from the astA gene-inserted pET-39b(+) cloned *E. coli* BL21(DE3). The expressed ASST was purified using His Bind column chromatography (2), and treated with enterokinase, and then separated by His Bind column chromatography (3). (1) indicates the molecular markers. (C) ASST purified from the astA gene-inserted pET-26b(+) cloned *E. coli* BL21(DE3). The expressed ASST was purified using His Bind column chromatography (2), dialyzed and rechromatographed on a His Bind column chromatography (3). (1) indicates the molecular markers.

The ASST expressed in *E. coli* BL21(DE3) was collected by centrifugation, sonicated, and then centrifuged again. The resulting supernatant was purified by His Bind column chromatography, and the eluted ASST expressed in pET 39b(+) vector was treated with enterokinase to exclude the DsbA and chromatographed on a His Bind column. Before the enterokinase treatment, the molecular weight of the ASST expressed with the pET-39b(+) and pET-26b(+) vectors was determined to be 100 kDa and 75 kDa. After the enterokinase treatment, the DsbA forward (approximately 25 kDa) of ASST expressed in the pET-39b(+) vector was cleaved. SDS-PAGE showed that the molecular weight of the cleaved

Table 2. Purification of the Arylsulfate Sulfotransferase Expressed from *astA* Gene Cloned *E. coli* BL21(DE3)

Stage	Activity (μmol/min)	Protein (mg)	Specific activity (μmol/min/mg)	Yield (%)
in <i>E. coli</i> BL21(DE3)-pET-39b(+)				
1. Crude extract	32	22	1.5	100
2. His-Bind column I	31.2	1.4	22.3	107
3. Enterokinase	20.5	0.8	25.6	64.0
in <i>E. coli</i> BL21(DE3)-pET-26b(+)				
1. Crude extract	35	14	2.5	100
2. His-Bind column I	28.6	0.8	35.8	81.7
3. His-Bind column II	26.0	0.7	37.1	74.3

ASST was approximately 75 kDa (Fig. 4). The specific activity of the purified enzyme expressed with the pET-39b(+) and pET-26b(+) vectors were 25.6 μmol/min/mg and 37.1 μmol/min/mg (Table 2). The specific activity of these purified ASSTs was low compared with that of the previously reported *Eubacterium* A-44.⁴⁾ In order to determine the significance of this observation, the effect of Ni²⁺ on the ASST activity was investigated. Ni²⁺ inhibited the enzyme but the inactivated enzyme activity was not reactivated by dialysis. These results suggest that the low specificity activity of the recombinant ASST may be due to the difference of ASST activity assay between the present and previous reports^{1,4)} or the inactivation of the overexpressed ASST by unknown factors.

The acceptor substrate specificity of the ASSTs purified from the cloned *E. coli* was investigated (Table 3). Among the substrates tested, tyramine was the best acceptor substrate of the cloned ASSTs, followed by tyrosine and 4-hydroxybenzoic acid. Their substrate specificity was similar to that of the ASST purified from *Eubacterium* A-44.²⁾

Overall, it is believed that the expressed ASST can be applied on an industrial scale to areas such as the sulfation of peptides.

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Table 3. Acceptor Substrate Specific Activity of Arylsulfate Sulfotransferases Expressed in *astA* Cloned *E. coli* BL21(DE3)

	Relative activity ^{a)} (%)		
	pET-39b(+)	pET-26b(+)	A-44
Tyramine	100	100	100
4-Hydroxy benzoic acid	3.6	4.3	2.3
Tyrosine	8.7	7.1	2.8

The specific activities of the purified enzymes, ASSTs inserted and expressed and purified in pET-39b(+) and in pET-26b(+) and purified A-44 for tyramine + PNS were 22.1, 37.1 and 75.5 μmol/min/mg, respectively, which are shown as 100%, respectively.

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