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Expression and Purification of a Functional Recombinant Aspartate Aminotransferase (AST) from *Escherichia coli*

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Aspartate aminotransferase (AST; E.C. 2.6.1.1), a vitamin B6-dependent enzyme, preferentially promotes the mutual transformation of aspartate and α-ketoglutarate to oxaloacetate and glutamate. It plays a key role in amino acid metabolism and has been widely recommended as a biomarker of liver and heart damage. Our study aimed to evaluate the extensive preparation of AST and its application in quality control in clinical laboratories. We describe a scheme to express and purify the 6His-AST fusion protein. An optimized sequence coding AST was synthesized and transformed into *Escherichia coli* BL21 (DE3) strain for protein expression. Ideally, the fusion protein has a volumetric productivity achieving 900 mg/l cultures. After affinity chromatography, the enzyme activity of purified AST reached 150,000 U/L. Commutability assessment between the engineered AST and standard AST from Roche suggested that the engineered AST was the better candidate for the reference material. Moreover, the AST showed high stability during long-term storage at −20°C. In conclusion, the highly soluble 6His-tagged AST can become a convenient tool for supplying a much better and cheaper standard or reference material for the clinical laboratory.

Keywords: Aspartate aminotransferase, enzyme activity, gene recombination, protein expression and purification, quality control

Introduction

Aspartate transaminase (AST), also known as aspartate aminotransferase or serum glutamic oxaloacetic transaminase, was the first purified transaminase family member [6]. This pyridoxal-phosphate-dependent transaminase enzyme represents a useful transformation of the amino group from aspartate or glutamate to the corresponding ketoacid. AST, as a dimer consisting of two identical subunits, has about 400 amino acid residues with a molecular mass of about 45 kDa [8]. The mammalian aspartate aminotransferase enzyme exists in two isoenzymic forms: mitochondrial (mAST) and cytosolic. These two isoenzymes are produced from an identical ancestral AST gene and share approximately 45% amino acid sequence homology [14].

High concentrations of AST are found in cells from the liver, heart, skeletal muscles, and red blood cells. Therefore, AST is commonly used as a clinical marker for liver diseases, particularly those with hepatocellular damage. AST is widely distributed and has been purified from a variety of sources in previous studies, including mammalian organs [2], plants [9, 17], bacteria [10], yeast [19], and green algae [11]. Currently, AST reference materials used in clinical laboratories are often obtained from human serum, which is costly, low-yield, and sometimes with poor specificity and stability [18]. In addition, most serum samples with high AST were from communicable hepatitis patients. The aim of the present study was to provide a higher concentration of soluble AST, supplying a better and cheaper reference material to the clinical labratory. It is therefore of interest to express and purify pure, soluble, and functional AST from *Escherichia coli (E. coli)*, and compare it with other commercially available ASTs.

E. coli is a widely used expression host. It grows in a simple, rapid, and economic manner and can provide a highly effective process for genetic engineering. Meanwhile,

the affinity tags have been increasingly used in recombinant expression. Recombinant proteins are often expressed in *E. coli*. They have small affinity tags such as hexahistidine (His-tag), which allow the desired protein to be efficiently separated by affinity chromatography [13]. The purification and detection of His-tagged proteins are easy under specific buffer conditions, since the string of histidine residues can bind to some immobilized metal ions such as nickel, cobalt, and copper.

In this paper, a codon-optimized sequence coding mAST was synthesized, and the mAST cDNA was inserted into the vector pRSFDuet-1. A sequence encoding six consecutive histidine residues (6His-tag) located at the 5′ end of the mAST-encoding gene was identified. The recombinant mAST was expressed in *E. coli* BL21 (DE3). Intact and biologically active mAST was obtained and most products existed in soluble form. The recombinant intact mAST was purified to homogeneity with a concentration of 0.9 g/l and a specific activity of 150,000 U/L. Furthermore, we observed that the recombinant mAST had good commutability with the standard enzyme of Roche. Following 64 weeks of storage, we examined the activity of stored mAST enzyme at different time points. We demonstrated that the mAST protein was stable with a long shelf-life. Likely, the pure target protein maintained its original biological and functional behaviors over an extended period of storage, which may range from weeks to years.

Materials and Methods

Materials

E. coli strains TOP10 and BL21 (DE3) used for gene manipulation and expression, respectively, were purchased from TIANGEN Biotech Co., Ltd. (Beijing, China). The vector pRSFDuet-1 for expressing 6His-mAST fusion protein was obtained from Novagen (Madison, WI, USA). Restriction enzymes (*Bam*HI and *Xho*I), T4 DNA ligase, and *Pfu* DNA polymerase were purchased from Takara Biotech Co., Ltd. (Dalian, China). Gel extraction and purification kits were from Axygen Scientific, Inc. (Union City, CA, USA). Oligonucleotides were synthesized by Invitrogen Biotech Co., Ltd. (Shanghai, China). Isopropyl β-D-1-thiogalactopyranoside (IPTG), Luria-Bertani medium, and kanamycin were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Codons Optimization and Expression Vector Construction

The rare codons of the mAST gene (NM_002080) were substituted by frequently used codons with the *E. coli* Codon Usage Analyzer 2.1, and then the optimized gene sequence (plus *Bam*HI and *Xho*I restriction enzyme sites) was synthesized. The obtained DNA fragment was digested with restriction enzymes, and cloned between the *Bam*HI and *Xho*I sites of 6His-pRSFDuet-1. The synthetic mAST gene was confirmed by DNA sequencing. The resulting plasmid, named 6His-mAST-pRSF, was transformed into *E. coli* BL21 (DE3) following the standard procedure.

Recombinant Expression and Cell Disruption

Overnight cultures of *E. coli* BL21 (DE3) cells from a single colony were inoculated at the ratio of 1% (volume) into Luria-Bertani medium, and then grown at 37°C for 3 h. IPTG was added to a final concentration of 0.5 mM at 25°C for 8 h of induction. Cells were collected by centrifugation at 3500 rpm for 10 min, and then suspended in 50 ml of cold lysis buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, and 10 mM imidazole) and disrupted by sonication. The cell lysates were then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant (soluble fraction) and pellet (insoluble fraction) were subjected to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to examine the expressed protein.

Extraction and Purification of Target Protein

Lysates were clarified by centrifugation and allowed to bind to nickel affinity resin (Ni-NTA superflow, Qiagen) for 1 h at 4°C with gentle mixing. After having been washed with lysis buffer, the mAST protein was eluted with elution buffer solution (10 mM NaH_2PO_4, 100 mM NaCl, pH 8.0, and 250 mM imidazole). The elution fractions were purified by a Sephadex G-50 column with an automated protein separation chromatography system (Jinhua Inc., Shanghai, China).

SDS-PAGE Analysis

Standard SDS-PAGE (12% gel) was applied for protein assay. The soluble proteins were mixed with an equal volume of 2× SDS sample loading buffer. The mixture was boiled for 5 min and added to a 12% separating gel, which was run at a constant voltage of 120 V. After electrophoresis, SDS-PAGE gels were stained with Coomassie Brilliant Blue G-250 (Bio Basic Inc., Toronto, Canada).

Analysis of Proteins

The protein concentration was measured by the Bradford protein assay. A small amount of protein sample was combined with the Coomassie Brilliant Blue G-250 and mixed well, and the absorbance was measured at 595 nm with a spectrophotometer (Molecular Devices Corp., CA, USA). Protein concentrations were estimated by comparison against a standard curve provided by standard protein dilutions, which were assayed alongside the samples. mAST enzyme activity was measured using the α-ketoglutarate reaction (Roche, Basel, Switzerland, 11876848) by a Roche modular automatic biochemical analyzer (Roche, Basel, Switzerland). For study on the commutability of recombinant AST protein, both the purified mAST in our lab and the standard aspartate transaminase from pig heart (Roche, Basel, Switzerland, 10105554001) were diluted with blood serum in various concentrations, and then the activities were determined.

Results

PCR-Based Synthesis of Codon-Optimized mAST Gene

Species-specific variations in codon usage are correlated with gene expression levels in the *E. coli*. The presence of rare codons can lower the translation rate and induce translation errors [1]. Therefore, we utilized codon optimization and synthesized the full-length codon-optimized gene to overcome this problem.

AST contains 430 amino acids, among which 73 amino acids (16%) can be encoded by the rare codons in *E. coli*. AGG, AGA, GGA, CGG, and CCC codons may be associated with the deleterious effects on heterogeneous protein expression in *E. coli*. In addition, two amino acids are encoded by AGA, which is considered as the least used codon in *E. coli* and will lead to a dramatic reduction of the target protein expression level (Table 1).

To minimize the rare codons' potential adverse effect on mAST expression in *E. coli*, these codons were altered with commonly used codons, and a new gene "AST" was designed for synthesis (Table 1). The underlines in Table 1 emphasize the swapped bases. The table of codon usage frequency in *E. coli* (http://www.faculty.ucr.edu/~mmaduro/codonusage/usage.htm) was followed when selecting the preferential codons. Using the robotic synthesis from pairs of overlapping oligonucleotides method [3], the 1,293 bp synthetic mAST gene was generated and cloned into the pRSFDuet-1 vector, and the construct was confirmed by DNA sequencing before transformation and expression.

Table 1. Comparisons of native codons with optimized codons in the mAST gene.

Position	Rare codon	Optimized codon	Position	Rare codon	Optimized codon
10 L	C T C	C T G	199 S	T C A	T C C
11 P	C C C	C C T	201 I	A T A	A T C
12 G	G G G	G G T	205 S	A G T	A G C
21 L	C T C	C T G	207 L	C T T	C T G
28 R	A G A	C G T	208 L	C T T	C T G
39 G	G G A	G G T	216 P	C C C	C C A
43 P	C C C	C C G	218 G	G G A	G G C
46 G	G G A	G G T	229 I	A T A	A T T
53 R	A G G	C G C	231 T	A C A	A C C
63 G	G G A	G G T	236 R	A G G	C G T
68 R	C G G	C G T	238 L	C T C	C T G
72 G	G G A	G G C	251 S	A G T	T C T
92 L	T T G	C T G	273 L	C T C	C T G
98 P	C C C	C C A	276 S	T C A	A G C
100 G	G G G	G G C	283 L	T T A	C T G
101 G	G G A	G G C	289 G	G G A	G G C
112 L	C T A	C T G	303 R	A G G	C G T
121 L	T T G	C T G	306 S	T C A	T C T
123 S	A G T	A G C	308 L	T T G	C T G
125 R	C G G	C G T	311 L	T T G	C T G
134 G	G G A	G G T	314 P	C C C	C C G
136 G	G G A	G G T	320 P	C C C	C C T
138 L	T T A	C T G	321 L	C T C	C T G
139 R	A G G	C G C	323 G	G G G	G G C
141 G	G G A	G G T	325 R	C G G	C G T
143 S	A G T	T C T	336 L	T T G	C T G
147 R	A G A	C G T	337 R	C G A	C G T
153 R	C G A	C G T	355 R	C G G	C G T
158 P	C C C	C C G	362 L	C T C	C T G
163 G	G G A	G G C	384 T	A C A	A C G
166 T	A C A	A C C	385 G	G G G	G G T
167 P	C C C	C C G	386 L	C T A	C T G
170 R	A G G	C G C	393 R	C G G	C G T
176 L	C T A	C T G	403 T	A C A	A C C
180 R	C G G	C G C	412 G	G G G	G G T
184 P	C C C	C C G	421 L	C T T	C T G
192 T	A C A	A C T			

Construction of the mAST Expression Plasmid

Fig. 1 shows the schematic representation of the 6His-mAST-pRSF expression plasmid. The mAST gene was controlled by the T7 promoter, and a 6His-tag was added at the N-terminal to promote the purification of target protein. The intact mAST consisted of 1,293 bp, with a calculated molecular mass of 45 kDa. Restriction enzymes *Bam*HI and *Xho*I were applied to digest the constructed expression vector; then we used agarose gel electrophoresis to verify the enzyme-digested product.

Expression and Purification of mAST Fusion Protein

The constructed expression vector, 6His-mAST pRSF, was transformed into *E. coli* BL21 (DE3). IPTG induced the expression of the 6His-mAST fusion protein. As shown in Fig. 2A, a band of 45 kDa was seen in samples of *E. coli* BL21, suggesting that the 6His-mAST fusion protein was successfully expressed. The 45 kDa mAST band appeared much stronger than the wild-type mAST. To further confirm that the 45 kDa band was mAST, SDS-PAGE was performed after affinity column chromatography (Fig. 2B).

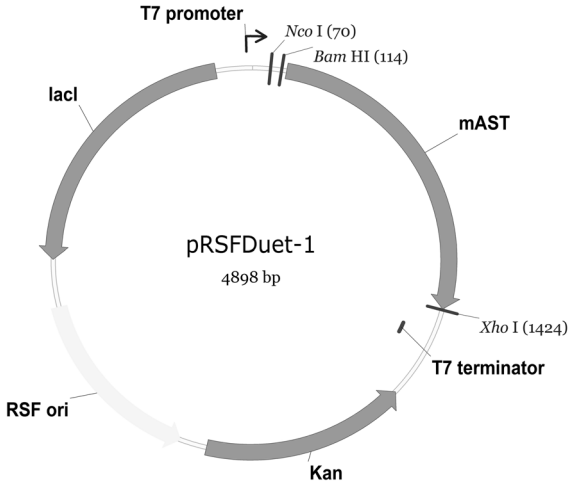

Fig. 1. Construction and verification of the recombinant expression plasmid 6His-mAST-pRSF.

Schematic diagram of the recombinant expression plasmid 6His-mAST-pRSF. The mAST gene was inserted between the *Bam*HI and *Xho*I sites of expression vector pRSFDuet-1 with the T7 promoter and a fusion partner of the N-terminal histidine affinity tag.

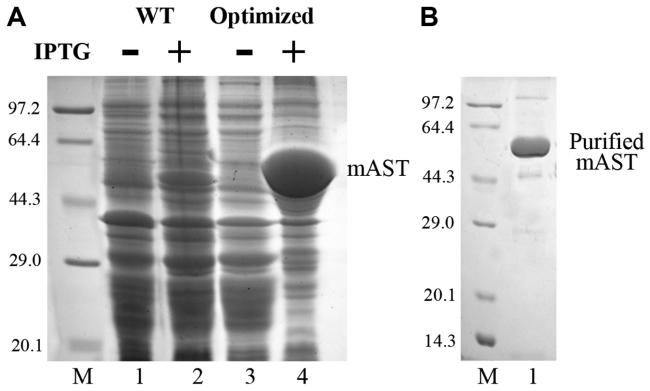

Fig. 2. SDS-PAGE analysis of total proteins and purified mAST.

(**A**) SDS-PAGE analysis of unpurified wild-type mAST and codon-optimized mAST. Lane 1: bacterial total proteins of wild-type mAST without IPTG induction; Lane 2: bacterial total proteins of wild-type mAST after IPTG induction; Lane 3: bacterial total proteins of codon optimized mAST without IPTG induction; Lane 4: bacterial total proteins of codon-optimized mAST after IPTG induction. (**B**) SDS-PAGE analysis of purified mAST protein by affinity chromatography. The visualized band of 45kDa was mAST.

Analysis of Protein Concentration, Enzyme Activity, and Commutability

It is well accepted that activities of proteins are closely related to their protein concentrations. We further examined the concentration of the AST fusion protein with the Bradford protein assay. During the processing of the protein samples and protein standards, both of them were mixed with assay reagent, and the absorbance was measured using a spectrophotometer. The concentration of the codon-optimized protein and wild-type protein tested by Bradford protein assay was 900 and 100 mg/l respectively. Enzyme activity was determined by the AST enzymatic assay kit of Roche, and the activity of purified mAST achieved to 150,000 U/L. In order to understand the commutability between the laboratory-purified mAST and the standard aspartate transaminase from Roche, total enzyme activities in a volume of 100 µl with various concentrations were measured. The standard curve was provided by linear regression analysis, and the high correlation coefficient ($r^2 = 0.999$) indicates that the recombinant mAST has good commutability with the standard enzyme of Roche (Fig. 3).

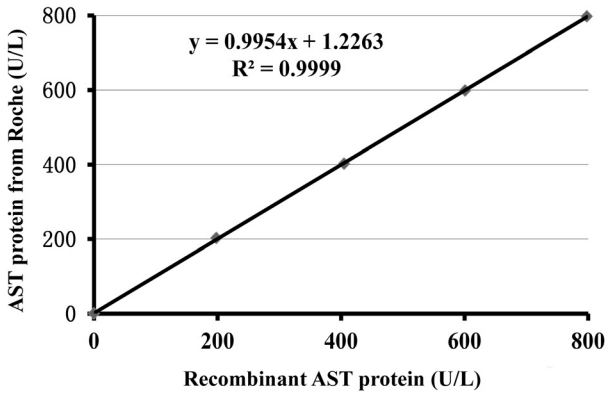

Fig. 3. Commutability between the purified mAST and the standard AST from Roche.

The regression line indicates that the activities of the serum-diluted recombinant mAST proteins were highly correlated with the standard proteins from Roche, in the range of 200–800 U/L.

Stability of AST Fusion Protein in Storage

Following the isolation and purification, it is often necessary to store the purified proteins for an extended period of time to evaluate their integrities and activities. The storage "shelf life" varies, ranging from several days to over one year, and depends on the protein nature and the storage conditions [5]. In order to investigate the stability of purified recombinant mAST protein stored in solution with 50% glycerol at –20°C, the enzyme activity was measured at different time points (0, 1, 2, 4, 8, 16, 32, and 64 weeks). As shown in Fig. 4A, all the samples with high-concentration (400 U/L), moderate-concentration (200 U/L) and low-concentration (40 U/L) mAST enzymes were stable with a long half-life, and the activity level was

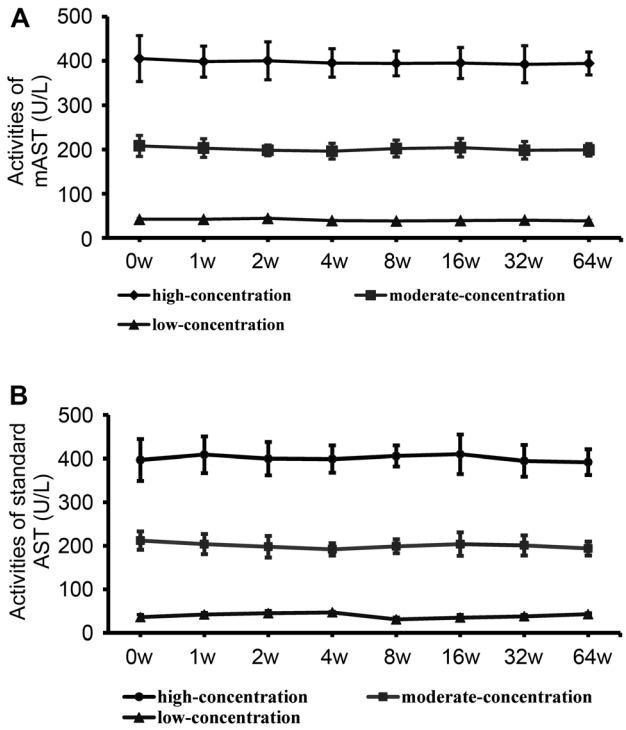

Fig. 4. Enzyme stability assay of the purified mAST and the standard AST from Roche.

(**A**) The high-, moderate-, and low-concentration purified mASTs were stored in solution (20 mM Tris-HCl, 10 mM KCl, pH 8.0) with 50% glycerol at –20°C, and then the enzyme activities were analyzed at the indicated time points. (**B**) The enzyme activities of standard ASTs from Roche were analyzed at the indicated time points.

decreased less than 3% after 64 weeks. The stability of the codon-optimized mAST was the same as the standard AST from Roche (Fig. 4B). These results suggest that the stability of the recombinant mAST was the same as the enzyme from the previous reports [15, 16], and the recombinant mAST was suitable for long-time storage at low temperature.

Discussion

AST is generally localized within the liver and heart, and the laboratory test for AST is helpful to identify damages in these organs. Mitochondria AST (mAST) and cytoplasmic AST are two distinct isoenzymes in human tissues [7]. mAST isoenzyme is present predominantly in liver, and the cytosolic isoenzyme is mainly from red blood cells and heart [12]. In patients with deteriorative liver problems, mAST can be readily released into the serum and therefore has more clinical significance than cytoplasmic AST.

Considering their cognate tRNA abundances in *E. coli*, many codons in mAST cDNAs are rarely used; and we can only routinely obtain a small volume of recombinant proteins upon the expressions in the forms of mAST recombinant in this organism. Thus, we have synthesized the gene, which is optimized for wild-typed human mAST proteins through its robotic synthesis from pairs of overlapping 40-mer oligonucleotides, to offer a more robust source of mAST enzymes to the clinical laboratory community [3]. In this study, the mAST synthetic gene was designed to be provided with hexa-his tags at the N-terminal end of mAST enzymes (sequences will be made available on demand). Meanwhile, we had engineered and successfully constructed the vector to produce 6His-tagged fusion mAST protein. The His-tag facilitated the purification of its fusion partners. After expression of the 6His-mAST fusion protein under IPTG induction, over 25% of the total *E. coli* protein was associated with the mAST band in Coomassie blue-stained SDS-PAGE gels (Fig. 2A). This expression level can be regarded as highly significant because, when using the correlative wild-type cDNA coding sequence without codon-optimized process, our previous expression systems had not induced any visible band in such SDS–PAGE gels of total *E. coli* cell protein.

Over the past decade, with the traditional preparation methods of reference material, AST reference materials used for quality control in clinical laboratories were commonly extracted from the serum samples of human beings, pigs, or other animals. This process is costly and complex; in particular, the use of human serum has a potential risk for spread of infectious diseases [4]. It is necessary to develop a simple and high-level expression system to generate a large amount of active, intact recombinant mAST enzyme in *E. coli*, and to greatly improve its applications in the clinical biochemistry laboratory with lower cost. In this study, we found that the activity of this recombinant mAST from the synthetic gene had similar aminotransferase activities as a standard commercially available AST enzyme from Roche, prompting that this high-concentration mAST is fully commutable with the standard mAST enzyme. Furthermore, this recombinant mAST showed great stability in long-term -20°C storage, which is an important feature for the reference material. In conclusion, this recombinant mAST possesses great potential to become a quality control enzyme for routine AST assay in the clinical laboratory.

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