Secretory expression of the rat aryl sulfotransferases IV with improved catalytic efficiency by molecular engineering

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Abstract

The rat aryl sulfotransferases IV (AST IV) has been used to catalyze 3'-phosphoadenosine-5'-phosphate (PAP) into the sulfonyl group donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) in biotechnological production of glycosaminoglycans. The performance of native AST IV is not satisfying due to the lower catalytic activity with substrate PAP. In the present study, we achieved secretory expression of the AST IV and improved its catalytic efficiency by molecular engineering. Fusion with signal peptides Cex, YebF and PelB allow for secretory expression of AST IV and the secreted AST IV yield reached 4.21 ± 0.23 U/mL, 8.67 ± 0.34 U/mL and 21.35 ± 0.87 U/mL, respectively. Modification of PelB further increased the secretory expression by more than fourfold, to 89.67 ± 1.34 U/mL. On this basis, molecular evolution of the predicted PAP-binding pocket gate loop was performed and a positive mutant L89S/E90L with higher activity was identified. Considering the importance of the sites Leu⁸⁹ and Glu⁹⁰, we performed site-saturation mutagenesis and found the mutant L89M/E90Q with much higher PAP affinity ($K_m = 0.46 \pm 0.02$ mM) and catalytic efficiency ($k_{cat}/K_m = 1816.32 \pm 12.72$ s/M). The secretory expression of the AST IV variant L89M/E90Q with higher catalytic efficiency should facilitate the studies on biosynthesis of sulfated polysaccharides.

Keywords Glycosaminoglycans · AST IV · PAPS · Molecular evolution · Sulfation

Introduction

Glycosaminoglycans, including heparin and chondroitin sulfate, have been widely used in the clinical treatment of antiinflammatory, antithrombin, osteoarthritis, and so on (Farrugia et al. 2018; Kang et al. 2018). Currently, the availability of heparin and chondroitin sulfate is still dependent on extraction from animal tissue. The animal source of

heparin and chondroitin sulfate can be easily contaminated by oversulfated chondroitin sulfate and kermatan sulfate which has caused several deaths (Guerrini et al. 2008). Enzymatic and chemoenzymatic synthesis of heparin and chondroitin sulfate are thought to be an alternative approach to produce a specific form of heparin and chondroitin sulfate without other GAGs' contamination (Kuberan et al. 2003; Zhou et al. 2018). However, the sulfonyl group donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) serves as the key cofactor for the sulfotransferases in the heparin and chondroitin sulfate biosynthesis pathway, and results in the production of byproduct, PAP, which is an inhibitor of sulfotransferases. Therefore, improving the regeneration efficiency of PAPS is crucial for the enzymatic and chemoenzymatic synthesis of heparin and chondroitin sulfate.

How to efficiently produce or regenerate PAPS in vitro has attracted intensive attention (Burkart et al. 1999; Zhou et al. 2018). In living cells, PAPS is biosynthesized from the energy compound ATP by two enzymes, ATP sulphydrylase and APS kinase, or by a dual functionality enzyme, PAPS synthetase (Supplement Fig. 1a) (Burkart et al. 2000; Rosenthal and Leustek 1995). In this regard, An et al. (2017)

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recently expressed the ATP sulfurylase from *Kluyveromyces lactis* and APS kinase from *Penicillium chrysogenum*, and constructed an enzymatic approach for producing PAPS with ATP as substrate. Alternatively, the one-enzyme regeneration system with a recombinant rat liver aryl sulfotransferase IV (AST IV) has been constructed for conversion of 3'-phosphoadenosine-5'-phosphate (PAP) to PAPS with the inexpensive compound *para*-nitrophenylsulfate (PNPS) as sulfate donor (Supplement Fig. 1b) (Burkart et al. 1999).

To date, many ASTs from human body (Kakuta et al. 1997) and bacteria, such as *Streptomyces* sp. (Kaysser et al. 2010) and *Escherichia coli* (Malojcic et al. 2008) have been identified and investigated. In particular, the human ASTs have been intensively studied and the substrate specificity and kinetic mechanism have been characterized (Campbell et al. 1987; Tyapochkin et al. 2009). A study of the enzyme structure demonstrated that the substrate-binding site of SULT1A1 (one human AST with 79.09% identity with rat AST IV) is plastic and is able to adapt its shape to accommodate different substrates (Gamage et al. 2005). Hence, these ASTs show broad substrate specificity, including tyramine, tryptosine methyl ester, epinephrine, and phenolic compounds such as naphthol, estradiol, phenol, flavone, chalcone, and xanthone (Duffel et al. 1998; Duffel and Jakoby 1981; Rao and Duffel 1991; van der Horst et al. 2015). Moreover, previous studies also showed that these ASTs exhibit highest affinity to naphthol, followed by estradiol, phenol, tyrosine methyl ester, tyramine, and epinephrine. PAP was not an ideal acceptor than low electron-withdrawing power substrates (for instance estradiol and phenol) (Duffel et al. 2001). Therefore, improvement of the affinity and catalytic efficiency of rat liver AST IV towards PAP should enable rapid sulfation of substrates of interest. In the present study, we aimed to achieve secretory expression of rat AST IV variants with higher affinity and catalytic efficiency towards PAP.

Materials and methods

Plasmids, strains and media

All the strains and plasmids used in this work are listed in Supplement Table 1. *E. coli* BL21 was used as the host for gene cloning and expressing. Codon-optimized rat AST IV gene for expression in *E. coli* was synthesized by GenScript (Nanjing, Jiangsu, China), and inserted into pColdIII plasmid (Takara Bio Inc., Shiga, Japan) after digestion with BamHI and XhoI to generate pColdIII-AST IV. The coding region of signal peptides, OmpA, Cex, PhoA, DsbA, AnsB, LamB, PgaA, YebF, PgaB, and PelB (Supplement Table 2) was amplified and fused to the 5' terminus of AST IV gene with Gibson assembly (Gibson et al.

2009). Luria–Bertani (LB) medium (NaCl 10 g/L, peptone 5 g/L, yeast extract 10 g/L) was used in all DNA manipulations. Modified LB (MLB, 4 g/L glycerol, 10 g/L NaCl, 5 g/L peptone, 10 g/L yeast extract) medium was used for protein overexpression. When necessary, 50 µg/mL ampicillin was added to provide selective pressure and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the expression of AST IV gene.

Mutation of PelB signal peptide to enhance protein secretion

Random mutagenesis of the PelB at the context of the cleavage sites was conducted by PCR using degenerated primers. In brief, the plasmid pColdIII-*pelB*-AST IV was amplified with primers *pelB*-MF (ATGGAA TTCTCTAGACCACCATTTGGTTCATG) and *pelB*-MR (NNNNNNNNCTGGGCAGCGAGGAGCAG. N stands for random nucleotides with equal frequency of A, C, G, and T), and then self-ligated using a Blunting Kination Ligation (BKL) Kit (Takara Bio Inc., Otsu, Japan), generating the mutation library of PelB. Clones carrying the PelB mutation library were grown in 96-well microtiter plates (200 µL MLB medium per well, Greiner Bio-One, Frick-enhausen, Germany) at 37 °C for 8 h using a microplate shaker HTS-S064 (Canvic, Shanghai, China). Then, they were transferred to new MLB broth in 48-well microtiter plates (1 mL MLB medium per well, Greiner Bio-One, Frick-enhausen, Germany). After that, they were cultivated on microplate shaker at 15 °C for 24 h. The culture supernatants were collected by centrifugation at 4000g, 4 °C for 20 min, and used immediately to AST IV activity assay. Positive mutants were sequenced by Sango (Shanghai, China).

Structure modeling of rat AST IV

Swiss model (<https://www.swissmodel.expasy.org/>) was used to construct the putative tertiary structure of rat AST IV with the crystal structure of human SULT1A1 as homologous template (PDB 3U3J, 2.7 Å resolution, the sequence has 79.09% identity comparing with rat AST IV) (Gamage et al. 2003). The interactions between enzyme and PAP were predicted with Discovery Studio 2017 software (Accelrys, Omaha, Nebraska, USA). The CDOCKER interaction energy between PAP and enzyme was then computed and the sizes of the substrate-binding pockets were calculated using the program CASTP (<http://cast.engr.uic.edu/cast/>) and the program POVME (Durrant et al. 2011). Structural diagrams were generated with PyMOL 1.8 (<http://pymol.sourceforge.net>).

Mutation of the PAP-binding pocket of AST IV

Based on the structure model of rat AST IV, the 11 amino acid residues Val⁸⁵-Pro-Ser-Gly-Leu-Glu-Thr-Leu-Glu-Glu-Thr⁹⁵, within 5 Å distance of the predicted PAP-binding pocket were selected for random mutagenesis. Primers containing degenerated nucleotides at the designated sites were designed, namely AST IV85-95F/AST IV85-95R, (Supplement Table 3). The plasmid pColdIII-*pelB*(M3)-AST IV was amplified with the above primers and self-ligated using a Blunting Kination Ligation Kit to create the mutation libraries of AST IV. Activity of AST IV mutants was measured in 96-well plated as described above. Positive mutants were sequenced by Sango (Shanghai, China).

Saturation mutagenesis of key amino acids involved in PAP binding

Saturation mutagenesis libraries of AST IV were constructed at sites A (Leu⁸⁹), and site B (Glu⁹⁰) with PCR using plasmid pColdIII-*pelB*(M3)-AST IV as template. The used primers are listed in Supplement Table 3. The template plasmids were degraded with DpnI at 37 °C for 2 h and PCR products were purified before transformed into *E. coli* BL21 (DE3). Saturation mutagenesis libraries were selected by ampicillin resistance on LB agar.

Purification of AST IV

Overnight culture was inoculated by 2% (v/v) into 50 mL MLB broth in 500 mL Erlenmeyer flask and incubated at 37 °C, 220 rpm. Expression of AST IV was induced by IPTG 2 h later. After that cultures were shifted to 15 °C and incubated for 24 h. The culture supernatants were collected by centrifugation at 8000g, 4 °C for 10 min, and filtered through a 0.22 µm membrane. AST IV was then purified with Ni-NTA agarose by affinity chromatography (GE Healthcare, Little Chalfont, UK). Briefly, the Ni-NTA agarose was incubated with the filtrated supernatant and washed thoroughly with washing buffer (20 mM Tris-HCl, pH 7.0, 30 mM imidazole, 500 mM NaCl). AST IV was released from Ni-NTA agarose with elution buffer (20 mM Tris-HCl, pH 7.0, 500 mM imidazole, 500 mM NaCl). Purified AST IV concentration was determined by Modified Bradford Protein Assay Kit (Sango, Shanghai, China) using bovine serum albumin as standard. Then, AST IV was adjusted to 10 g/L in 20 mM Tris-HCl (pH 7.0) and stocked for kinetic and thermodynamics parameter assay.

AST IV activity analysis

AST IV activity was determined by measuring the light absorbance at 400 nm, which is in correlation with the

formation of free *p*-nitrophenol (Yang et al. 1996). Briefly, 20 µL 500 mM *p*-nitrophenyl sulfate (PNPS) and 0.1 mM PAP solution (100 mM Tris-HCl buffer, pH 7.0) were added into 180 µL culture supernatants with the total reaction volume set to 200 µL. The reactions were monitored in 96-well microtiter plates (Sigma, St Louis, MO, USA) at 37 °C for 15 min. One unit of activity was defined as the amount of enzyme needed to catalyze the formation of 1 µM-PNP at 37 °C within 1 h. Specific activity was expressed in unit/mg protein.

Analysis of the AST IV enzyme kinetics and thermostability

For kinetic assay of AST IV, 20 µL purified AST IV (10 g/L) was added into 980 µL of 20 mM Tris-HCl (pH 7.0) solution containing 50 mM PNPS, and 0.004–2.12 mM PAP. The total reaction volume was set to 1 mL in 1.5 mL EP tubes. *p*-Nitrophenol formation was monitored by measuring the absorbance at 400 nm every 2 s. Kinetic parameters of AST IV was determined using the initial rate of *p*-nitrophenol formation and calculated according to Eadie-Hofstee plots by nonlinear repression (Weinstock et al. 1981).

Statistical analysis

Values are expressed as mean ± SD. Mean value comparisons between two groups were performed by *t* test, and significant differences were considered at *p* < 0.05.

Results and discussion

Signal peptide screening for AST IV secretion

Although the PAPS regeneration system that depends on AST IV has been constructed and applied for biosynthesis of heparin and chondroitin sulfate polysaccharides, intracellular expression with low activity (37.62 ± 0.8 U/mL) is still a bottleneck for its practical applications (Zhou et al. 2018). Here, to facilitate downstream purification, we first focused on the secretory expression of AST IV. To this end, the commonly used signal peptides OmpA, Cex, PhoA, DsbA, AnsB, LamB, YebF, PgaB, PgaA and PelB (Fig. 1a) were investigated for AST IV secretion. As shown in Fig. 1b and c, YebF, Cex and PelB successfully secreted AST IV into culture and AST IV achieved the maximum enzyme activity value (21.35 ± 0.87 U/mL). To further increase the protein secretion, the cleavage region (c-region) of PelB for translocation of target proteins through cell membrane was engineered via random mutagenesis. After screening in 96-well microliter plates, three positive mutants AST IV M1 (AQA), AST

Fig. 1 Effects of different signal peptides on the secretion of ASTIV by *E. coli*. Artificial operons constructed for the secretion of ASTIV (a). Effect of different signal peptides on the secretion of ASTIV by *E. coli* (b). SDS-PAGE analysis of the expression of ASTIV (c): 1:

supernatant of *E. coli* BL21 pColdIII-*pelB*(M1)-ASTIV; 2: supernatant of *E. coli* BL21 pColdIII-*pelB*(M2)-ASTIV; 3: supernatant of *E. coli* BL21 pColdIII-*pelB*(M3)-ASTIV; 4: supernatant of *E. coli* BL21 pColdIII; M marker

IV M2 (ASA) and AST IV M3 (AGA) that increased secretory expression of AST IV to 32.02 ± 0.63 U/mL, 64.05 ± 1.50 U/mL and 89.67 ± 1.34 U/mL, respectively, were identified (Fig. 1b). Efficient secretory expression of AST IV M3 (AGA) would contribute to the increased AST IV production, which was 2.38 times of intracellular expression, simplifying downstream process.

Molecular modeling of AST IV substrate-binding pocket

AST IV catalyzed the transfer of a sulfonate group from PNPS to the substrate, PAP. Therefore, two essential components of its catalytic action are the PNPS-binding region, and PAP-binding region. To identify the potential targets sequence for engineering to improve the AST IV activity, the protein sequence of AST IV was aligned with the sequences of human SULT1A1, SULT1A2 and SULT1A3. A molecular structure model of AST IV was constructed for the docking analysis of PAP with the Swiss Model program with the structure of human SULT1A1 as template (Fig. 3a). As shown in Fig. 2, consistent with human sulfotransferases, the active site His¹⁰⁴ is highly conserved in the rat AST IV. Moreover, the residues Phe²⁰, Phe⁷⁷, Phe⁸⁰, Lys¹⁰², and Tyr²³⁶ that are involved in the PAP-binding pocket formation are also conserved in AST IV (Fig. 3b). In addition, the gating loop Lys⁸¹-Cys-Pro-Gly-Val-Pro-Ser-Gly-Leu-Glu-Thr-Leu-Glu-Glu-Thr⁹⁵ controlling the entrance and evacuation of non-sulfonated substrates and sulfonated products (Berger et al. 2011; Lu et al. 2010) is also conserved (Fig. 3a, highlighted).

Modification of the gating loop for identifying crucial residues towards PAP binding and sulfur transfer activity

On the basis of molecular modeling analysis, the motif Val⁸⁵-Pro-Ser-Gly-Leu-Glu-Thr-Leu-Glu-Glu-Thr⁹⁵ that is located in the gating loop and within 5 Å distance around the PAP-binding pocket was selected and randomly mutagenized. After ten rounds of mutagenesis and screening (about 12,000 transformants were screened to ensure a reasonable coverage of the corresponding amino acid residues.), a positive variant L89S/E90L with significantly increased enzyme activity (112.09 ± 1.57 U/mL) was obtained (Fig. 4a). The increased enzyme activity could be attributed to the alternation of enzyme catalytic efficiency, since no obvious increase of the mutant expression was observed (data not shown). To characterize the kinetic parameters, the wild-type AST IV and the variant L89S/E90L were purified and examined (Fig. 4b). Compared with the wild-type AST IV, the variant L89S/E90L showed a decreased K_m value (0.90 ± 0.11 mM) and significantly increased k_{cat} (0.36 ± 0.01 /s) and V_{max} (117.36 ± 2.53 μM/min) values (Fig. 4b). As a consequence, a much higher catalytic efficiency (k_{cat}/K_m) of L89S/E90L was obtained (400.48 ± 5.93 /s/M) which was 2.75-fold of that of the wild-type AST IV (145.67 ± 7.56 /s/M) (Fig. 4b). The results indicate that residues Leu⁸⁹ and Glu⁹⁰ should play crucial roles in interactions between PAP and the binding pocket, because we found CDOCKER interaction energy of mutant L89S/E90L (-15.43 kcal/mol) is lower than that of the wild type (-13.68 kcal/mol). That means Leu⁸⁹ and Glu⁹⁰

Fig. 2 Structure-based sequence alignment of rat ASTIV and human SULT1As. Secondary structural elements in SULT1As are indicated by yellow arrow (strand) and green cylinder (helix) above the

sequence. Residues shown in red color are the substrate-binding residues of ASTIV. Residues shown in the dash rectangle are the region for random mutation

Fig. 3 Structure model of ASTIV. Homology-modeled rat ASTIV (cartoon) complexed with PAP (sticks) by the Swiss Model program (a). Stereo drawing of the autodocking analysis of PAP analysis (b).

The residues in blue color are the substrate-binding residues; the residues in red are the gate to substrate-binding pocket

are important positions that control the conformational changes of AST IV during binding of substrate. Therefore, the mutant L89S/E90L has more affinity to the

substrate PAP than that of control. Furthermore, the results also confirmed previous studies that introduction of aliphatic amino acid residues near the binding pocket

Fig. 4 Effect of site mutagenesis on the catalytic activity. Effect of saturation mutagenesis on Leu⁸⁹ and Glu⁹⁰ on the ASTIV activity (a). Effect of mutagenesis on L89 and E90 on the kinetic parameters of ASTIV (b)

decreases the repulsive forces between enzyme and PAP and results in improved substrate affinity and catalytic efficiency (Brix et al. 1999; Dajani et al. 1998; Liu et al. 2000; Sakakibara et al. 1998).

Site-saturation mutagenesis of Leu⁸⁹ and Glu⁹⁰ for improving substrate affinity and catalytic efficiency

In consideration of the positive effects of double mutation of L89S/E90L on PAP-binding affinity and the catalytic efficiency, we did site-saturation mutagenesis to the residues Leu⁸⁹ and Glu⁹⁰. The variants L89E, L89I, L89F, L89A, L89T, L89Y, L89Q, L89S, E90C, and especially L89 M and E90Q showed increased activities (Fig. 4a) compared to the wild-type AST IV (Fig. 1c). Hence, the mutation L89 M and E90Q were combined to generate the variant L89 M/E90Q. This mutant displayed the highest culture activity of 157.83 ± 3.94 U/mL (Fig. 4a). To explore what makes the AST IV variant L89M/E90Q more efficient in regenerating PAPS, we purified this enzyme and analyzed its kinetic parameters. As shown in Fig. 4b, compared with the wild-type AST IV, the variant L89M/E90Q exhibited much higher PAP affinity ($K_m = 0.46 \pm 0.02$ mM, CDOCKER interaction energy = -19.48 kcal/mol) and catalytic efficiency ($k_{cat}/$

$K_m = 1816.32 \pm 12.72/\text{s}/\text{M}$, 12.46 times than wild type). The results confirmed that the significantly increased activity of AST IV could be ascribed to the improved catalytic efficiency, since no increased expression was observed (data not shown).

Conclusions

In the present study, by engineering signal peptide and the gating motif of rat AST IV, we successfully achieved secretory expression of AST IV with improved catalytic efficiency. In particular, fusion with the modified signal peptide PelB resulted in efficient secretion of AST IV (89.67 ± 1.34 U/mL). By molecular engineering, we constructed the positive mutant of AST IV, L89 M/E90Q. This mutant has been secreted successfully by the modified PelB and the measured enzymatic activity within the medium reached 157.83 ± 3.94 U/mL, which was 4.20-fold of the highest intracellular expression level in *E. coli* (37.62 ± 0.8 U/mL). Efficient secretory expression of the rat AST IV variant with higher catalytic efficiency ($k_{cat}/K_m = 1816.32 \pm 12.72/\text{s}/\text{M}$, which was more than tenfold of that of the wild-type AST IV) would not only accelerate related studies on biosynthesis of polysaccharides such as heparin and chondroitin sulfate but could also decrease the production cost.

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Author contributions ZZ and ZK designed the experiments. QL, RX, and BW carried out all the experiments and analyzed data. GD wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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