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Overexpression of Arylsulfatase in *E. coli* and Its Application to Desulfatation of Agar

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Abstract The arylsulfatase gene (*astA*, 984 bp ORF) from the *P. carrageenovora* genome was amplified by PCR and subcloned into the pET21a vector. When the constructed plasmid pAST-A1 (6.4 kb) was introduced into *E. coli* BL21(DE3), the transformant on the LB plate containing IPTG showed a hydrolyzing activity for 4-methylumbelliferyl sulfate and *p*-nitrophenyl sulfate. The highest arylsulfatase activity (2.1 unit/ml) was obtained at 10 mM IPTG. Most arylsulfatase activity was found in the cell lysate, whereas no significant activity was detected in the culture supernatant. The molecular weight of the recombinant enzyme was estimated to be 33.1 kDa by SDS-PAGE. After the reaction of agar with arylsulfatase for 12 h at 40°C, the gel strength of the agar increased by 2-fold, and 73% of the sulfate in the agar had been removed. This result suggests that arylsulfatase expressed in *E. coli* could be useful in the production of electrophoretic grade agarose.

Key words: Agar, arylsulfatase, desulfatation, *E. coli*, overexpression, *Pseudoalteromonas carrageenovora*

Agar is a structural polysaccharide found in the cell wall of red algae such as *Gelidium* and *Gracilaria* and is generally considered to be a mixture of about 70% agarose and 30% agaropectin [11]. Agarose consists of a repeating unit of alternating 1,4-linked 3,6-anhydro-α-L-galactosyl residues and 1,3-linked α-D-galactosyl residues, which can be named as agarobiose [1, 19, 25]. The structure of agaropectin is like agarose but additionally contains sulfate groups and carboxyl groups [1]. Enzymatic hydrolysis of sulfate groups in agaropectin simplifies the process of agarose preparation. To produce a value-added agarose from agar, the prerequisite is the supply of a large amount of arylsulfatase [28]. Incorporation of sulfate groups in agarose residues causes weakened gel strength due to the avoidance of a cross-linked structure during gelation [2].

Arylsulfatase (arylsulfate sulfohydrolase, EC 3.1.6.1), an enzyme involved in the metabolism of organic sulfur and sulfate, hydrolyzes arylsulfate esters to aryl compounds and inorganic sulfate. Sulfonated polysaccharides are abundant in the marine environment and work as a structural component in marine plants [20]. In the marine environment, which is abundant in sulfate ions [15, 29–31], arylsulfatase activity of *Alcaligenes metalcaligenes*is is not inhibited by sulfates [15]. However, bacterial arylsulfatase in the gut of marine fish and shellfish was inhibited by sulfate salts and phosphate salts [8]. Several marine animals that feed on algae are known to secrete carbohydrate sulfatases as digestive enzymes and cleave the sulfate ester bonds in dietary polysaccharides to improve digestion and the absorption of marine polysaccharides [14]. The digestive glands of various molluscs were found to be a rich source of carbohydrate sulfatase [22]. There have been several studies reporting that various arylsulfatases have been isolated from bacteria such as *Klebsiella pneumoniae* [21], *Salmonella typhimurium* [13, 24], *Serratia marcescens* [23], and *Pseudomonas aeruginosa* [6]. Also, the activities of arylsulfatase produced by bacteria, algae, and higher animals are known to depend on the formylglycine residue in the active sites [4, 12, 27]. However, *Pseudoalteromonas carrageenovora* arylsulfatase is considerably smaller (36 kDa) than the other bacterial sulfatases, and does not display sequence similarity to any of the sulfatase referred to above [15]. It shows a broad optimum in its activity around pH 8.5. The enzyme appears to be located in the periplasm, and is probably involved in the desulfatation of sulfated

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polysaccharides [3]. A marine aerobic Gram-negative bacterium, *P. carrageenovora* has been known to hydrolyze carrageenans, the sulfated galactans of red algae, and to desulfate oligo kappa-carrageenans. Recently, the gene encoding arylsulfatase of *P. carrageenovora* was cloned and the nucleotide sequence was reported [3]. To the knowledge of the researchers of this report, this is the first report about the functional expression of *P. carrageenovora* arylsulfatase in *E. coli* and its application to the production of agarose from agar.

This report describes the cloning and expression of the arylsulfatase of *P. carrageenovora* in *E. coli*, and the use of the recombinant enzyme in the production of a desulfated agarose.

MATERIALS AND METHODS

Strains and Plasmids

E. coli host strains used in this work are *E. coli* BL21 [(*DE3*), *F-*, *ompT*, *rB-*, *mB-*] and *E. coli* DH5α [*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lacZ* M15]. Genomic DNA of *Pseudoalteromonas carrageenovora* ATCC 43555 was used for the source of the *astA* gene. For the construction of an *E. coli* expression plasmid, the plasmid pET21a (5,443 bp, Novagen, Germany) was used, in which the *E. coli* T7 promoter and *lac* operator were connected. The *E. coli* vector pBluescript II SK(+) (2,961 bp) was also used for the subcloning and expression of the *astA* gene.

Media and Culture Conditions

P. carrageenovora and *E. coli* were grown on the ZoBell's 2216e medium (cultivating heterotrophic marine bacteria, Difco, U.S.A.) and Luria-Bertani (LB) medium, respectively. For the selection of *E. coli* transformants, LBAIM medium (LB, 30 μg/ml ampicillin, 1 mM IPTG, 5-100 mM 4-methylumbelliferyl sulfate) was used.

Construction and Transformation of Arylsulfatase Expression Plasmid

The *astA* gene of 0.9 kb was amplified by PCR using genomic DNA of *P. carrageenovora* as a template [3]. For the in-frame connection with the multicloning site of pET21a, two PCR primers were designed so as to have proper restriction enzyme sites at the 5' and 3' ends, and synthesized as the following sequences: 5'-CGGGATCC-CATGCAATTAGTATTATA-3' and 5'-CCAAGCTTTTAGC GTTTTAGTTCGTAAC-3'. After PCR amplification, the *astA* gene was treated with *Bam*HI and *Hin*dIII and then subcloned into the pET21a vector, resulting in pAST-A1 plasmid.

Analyses

E. coli cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). The *E. coli* culture broth was centrifuged, and then the supernatant was used for the measurement of extracellular arylsulfatase activity. The soluble and insoluble fractions of *E. coli* were obtained by centrifugation (8,000 ×*g* 10 min) of ultrasonic lysates. Arylsulfatase activity was assayed by measuring the amount of *p*-nitrophenol (*p*NP) released from 50 mM of *p*-nitrophenyl sulfate (*p*NPS) at pH 8.5 and 40°C. One unit of the enzyme activity was defined as the amount of enzyme liberating 1 μmole *p*NP from *p*NPS per minute. Protein electrophoresis was perfomed by SDS-PAGE (10% gel). For the zymogram analysis of the arylsulfatase, SDS in the gel was removed by shaking SDS-PAGE gel in 20 mM Tris-HCl (pH 8.5) at 4°C for 4 h, followed by adhesion with 1% agarose containing 5-50 mM 4-methylumbelliferyl sulfate (MUFS) at 37°C for 10-30 min.

Desulfatation of Agar with Arylsulfatase

The enzyme reaction was performed at 40°C for 12 h. The reaction mixture was composed of 7 g of agar (Junsei, Japan) and 1 l of 50 mM Tris-HCl buffer (pH 8.5). That was liquefied at 121°C for 15 min, and then reacted with the indicated amount of enzyme (1 unit per g agar) (filtrated cell lysates). Most of the water in the reaction solution was removed by freezing and thawing, followed by freeze drying. Gel strength was measured by Rheometer (Compac-100, Sun Scientific, Japan) and sulfate content was determined by and Dodgson and Price's method [10].

RESULTS AND DISCUSSION

Construction of Arylsulfatase Expression Plasmid

Arylsulfatase (*astA*) of *P. carrageenovora* was cloned and its *E. coli* expression plasmid (pET21a), pAST-A1 (6.4 kb), was constructed, in which the *astA* gene with its own

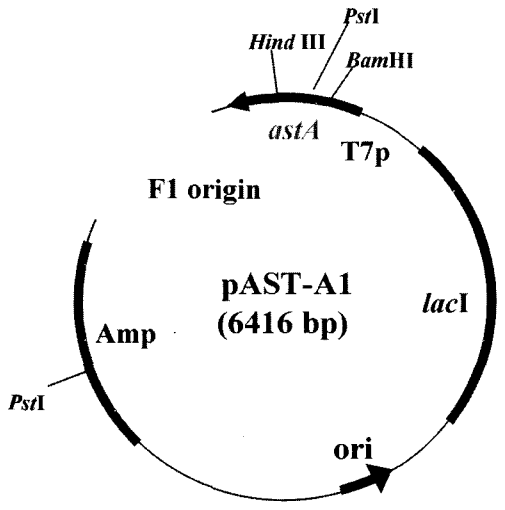

Fig. 1. Schematic diagram of arylsulfatase expression plasmid, pAST-A1.

ignal sequence (25 amino acids) was under the control of the T7 promoter (Fig. 1). Before this was done, the open reading frame (ORF) of the *astA* gene of *P. carrageenovora* was amplified and then subcloned into the pBluescript II SK(+). However, when the resulting plasmid was introduced into the *E. coli* DH5α cell, a low level of arylsulfatase activity (<0.3 unit/ml) was detected, both intracellularly and extracellularly, and the transformants showed the cell lysis (OD_{600}<0.3).

Overexpression of Arylsulfatase in *E. coli*

When the transformed cells were grown on an LBAI agar plate containing MUFS (4-methylumbelliferyl sulfate), only the *E. coli* BL21 (DE3) harboring pAST-A1 showed an intense fluorescence at 360 nm due to the liberated 4-methylumbelliferone (Fig. 2). This result indicates that the arylsulfatase gene was successfully expressed in *E. coli*.

P. carrageenovora synthesizes an arylsulfatase and locates it in a periplasmic space or external cell membrane [3]. In recombinant *E. coli*, the location of arylsulfatase varied according to the induction condition. When the transformant was fractionated into the periplasmic space and cytoplasm, the activity was detected in both fractions. Most activity was detected in the periplasmic space, as like in the expression of *Pyrococcus furiosus* Pfu DNA polymerase [5] and *Thermus caldophilus* alkaline phosphatase [17].

The effect of IPTG concentration on cell growth and arylsulfatase expression was investigated by varying the IPTG concentration from 0-5,000 μM. Then, the recombinant *E. coli* BL21 (DE3)/pAST-A1 was cultivated on LB medium containing 30 μg/ml ampicillin at 37°C and IPTG was added at the mid-exponential phase (OD_{600}=0.5-1.0). As shown in Fig. 3, at 10 μM IPTG most of the arylsulfatase activity was found in the cell lysate, and the highest arylsulfatase activity obtained was 2.1 unit/ml. However, at 50-5,000 μM IPTG concentration, activity was observed both in the culture supernatant (>50%) and the cell lysate (<50%). Cell growth gradually decreased until 50 μM IPTG. The overexpression of the arylsulfatase by excess IPTG may thus be harmful to *E. coli* and inhibit cell growth. This result was often observed in the expression of heterologous genes in *E. coli*. In the expression of *B. subtilis*

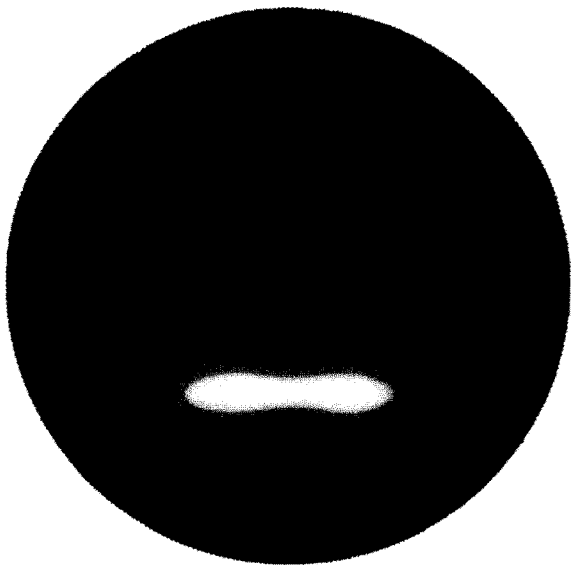

Fig. 2. Arylsulfatase activity of *E. coli* transformant. The cells were grown on LB medium containing 100 mM MUFS and 1 mM IPTG.
Symbols: **A**, *E. coli* DH5α/pAST-A1; **B**, *E. coli* BL21(DE3)/pET21a; **C**, *E. coli* BL21(DE3)/pAST-A1.

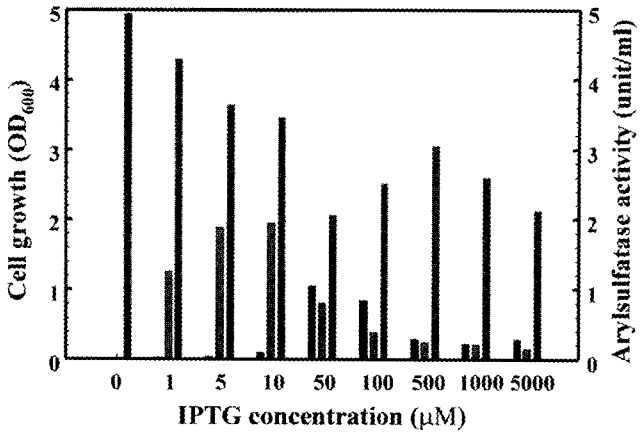

Fig. 3. Cell growth and arylsulfatase activity at 8 h after IPTG induction in *E. coli* BL21 (DE3)/pAST-A1. The IPTG solution with various concentrations was added at 2 h of culture, and the cell were harvested after 8 h from IPTG induction.
Symbols: (■), Arylsulfatase activity in culture supernatant; (▩), Arylsulfatase activity in soluble fraction of cell lysate; (▩), Cell growth.

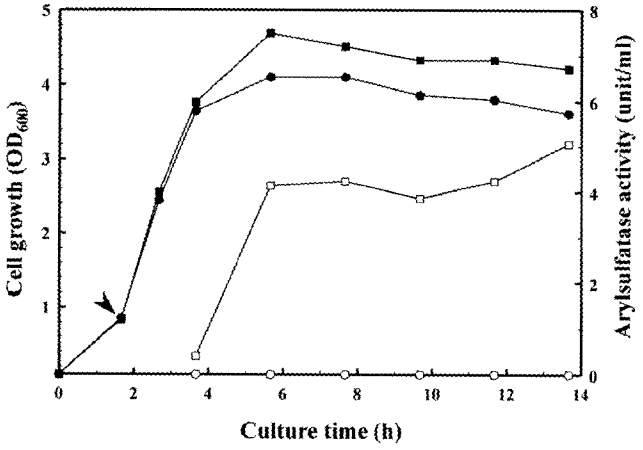

Fig. 4. Comparison of the cell growth and arylsulfatase activity between *E. coli* BL21/pAST-A1 and BL21/pET21a 10 μM IPTG was added at the early exponential phase (arrow head).
Closed and open symbols indicate cell growth and arylsulfatase activity, respectively. Symbols: (●, ○), BL21(DE3)/pET21a; (■, □), BL21(DE3)/pAST-A1.

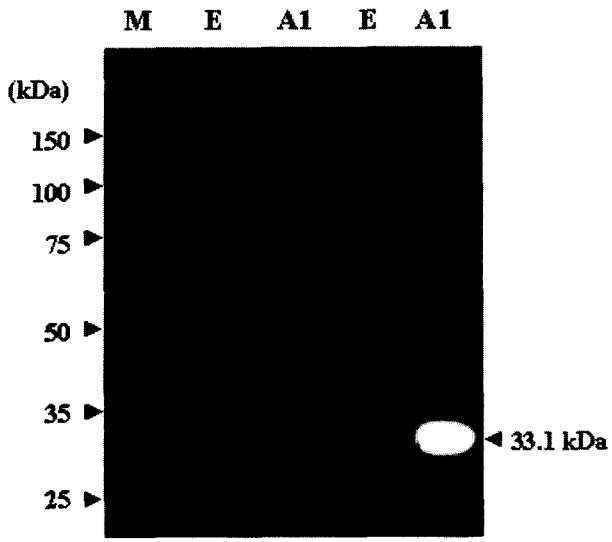

Fig. 5. Zymogram analysis of the recombinant arylsulfatase by staining with MUFS.
Symbols: **M**, marker; **E**, cell lysate of *E. coli* BL21(DE3)/pET21a; **A1**, cell lysates of *E. coli* BL21(DE3)/pAST-A1.

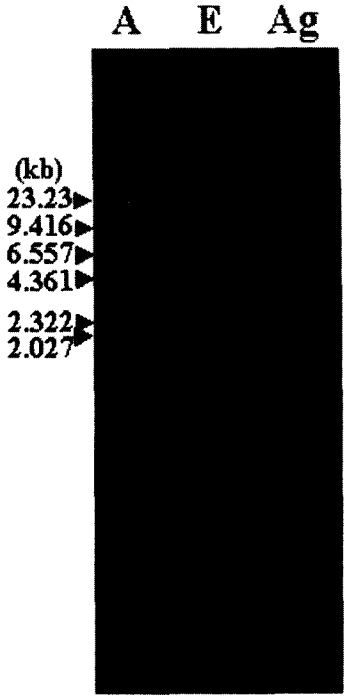

Fig. 6. DNA electrophoresis on gels from agar, commercial agarose, and agarose produced by the enzymatic method.
Symbols: **A**, Junsei agar; **Ag**, Invitrogen agarose; **E**, enzyme-treated agarose. 1% gel and 0.4 μg DNA of *Hin*dIII marker were used.

pectate lyase in *E. coli*, the localization or secretion of the gene product varied according to the inducer concentration [19]. In addition, cell lysis may affect the distribution of arylsulfatase activity [19]. The concentration of IPTG also changed the expression level of cyclodextrin glucanotransferase [16] and chlorella toxin A2B subunit in *E. coli* [18].

By the addition of 10 μM IPTG at a cell concentration of OD_{600}=0.85, arylsulfatase activity was maintained at a level of 4–5 unit/ml after 4 h of cultivation (Fig. 4). This arylsulfatase activity increased 2-fold more than that in the test-tube culture.

Zymogram Analysis of Recombinant Arylsulfatase

The arylsulfatase protein band on SDS-PAGE was not observed clearly with the cell lysate of *E. coli* BL21 (DE3)/pAST-A1. Therefore, the arylsulfatase band was comfirmed by zymogram analysis with MUFS. As shown in Fig. 5, the molecular weights of the recombinant arylsulfatase in the cell lysates were estimated to be 33.1 kDa by SDS-PAGE. This means that the signal peptide (25 amino acids) was correctly cleaved after expression.

Desulfatation of Agar by Arylsulfatase

After reaction of agar with arylsulfatase for 12 h at 40°C, the gel strength of the agar was increased 2-fold, and 73% of sulfate in the agar was removed (Table 1). The gel strengths of agar, enzyme treated agarose, and commercial agarose were about 250.0, 487.7, and 883.7 g/cm^2, respectively. The electrophoresis pattern of the DNA marker showed a similar migration and resolution for both the enzyme-treated agarose and the commercial one (Fig. 6), but the agar gel showed a slow migration as well as a low resolution. From this result, the existence of sulfate groups and cations in the agar was suspected to cause steric hindrances on DNA mobility, resulting in interruption of the migration and resolution of DNA [7].

Conventional methods for commercial agarose production are limited to the separation of agarose and agaropectin by using their solubilities in organic solvents such as polyethylene glycol [9, 26]. The solvent method greatly increases agarose loss during separation [22]. Thus, separation of agarose from agar with organic solvents is a laborious and time-consuming process resulting in high production cost. The recombinant arylsulfatase produced in this study can enhance the gelling strength of agar by removing sulfate

Table 1. Physical and chemical properties of agarose prepared by treatment of recombinant arylsulfatase.

1% Gel	Gel strength (g/cm^2)	Sulfate (%)	Yield (%)
Control (agar)	<250	>0.9	100
Enzyme-treated agarose (1 unit/g)	487.7±0.17	0.25±0.04	74.8±2.0
Commercial agarose (Invitrogen Co.)	883.7±0.13	<0.15	100

groups in agar, and a longer reaction may significantly increase the conversion rate of agar to agarose. Also, the enzyme could be applicable to the production of value-added products such as electrophoretic-grade agarose and chromatographic resin.

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