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Overexpression of Recombinant Arylsulfatase Cloned from *Pseudoalteromonas carrageenovora*

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Arylsulfatase cloned from a marine bacterium, *Pseudoalteromonas carrageenovora*, was overexpressed in *Escherichia coli*. Most of the recombinant arylsulfatase was found in the cell lysate with induction up to 10 μM IPTG. However, enzyme activity was observed both in the culture supernatant and cell lysate by induction with IPTG concentration of 50-5,000 μM. Most of the recombinant enzyme was localized in the periplasmic space with 10 μM IPTG induction, while half of the enzyme was distributed in the periplasmic space with 50 μM IPTG induction. Cell growth and arylsulfatase activity did not change with the induction time, and the level of recombinant arylsulfatase expression was maintained at 4-5 U/mL after 6 to 14 hr of culture.

Key words: Agar, Arylsulfatase, *Pseudoalteromonas carrageenovora*

Introduction

Agar is a structural polysaccharide found in the cell walls of red algae, such as *Gelidium* sp. and *Gracilaria* sp., and is a mixture of approximately 70% agarose and 30% agaropectin. 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 are called agarobiose. The structure of agaropectin is like that of agarose, but it also contains sulfate groups in the C6 position of the galactosyl residues, forming L-galactose-6-sulfate. The incorporation of sulfate groups in agarose residues reduces gel strength because it prevents formation of a double helix structure during gelation.

Arylsulfatases (arylsulfate sulfohydrolase, EC. 3.1.6.1) hydrolyze arylsulfate ester bonds into the corresponding phenols and inorganic sulfate. Arylsulfatases are found in a wide range of organisms, from mammals to bacteria, and their primary structures are very similar in different species (Kertesz, 1999). Most studies of microbial arylsulfatase have focused on pathogenic bacteria, such as *Klebsiella pneumoniae* (Miech et al., 1998), *Salmonella typhimurium* (Henderson and Milazzo, 1979), *Pseudomonas aeruginosa* (Beil et al., 1995), and *Escherichia coli* (Jansen et al., 1999). In the marine environment, Arylsulfatase activity has been reported from algae that facilitate the assimilation and dissimilation of sulfate in the cell (De Hostos et al., 1988). The digestive glands of various mollusks are a rich source of arylsulfatase (Milanesi and Bind, 1972). Several marine animals that feed on marine plants secrete carbohydrate sulfatases as digestive enzymes and cleave the sulfate ester bonds in dietary polysaccharides to improve the digestion and absorption of marine polysaccharides (Hoshi and Moriya, 1980). An arylsulfatase-producing marine bacterium was isolated and identified as *Pseudoalteromonas carrageenovora* (Akagawa-Matsushita et al., 1992) and its gene was cloned (Barbeyron et al., 1995). Arylsulfatase from *P. carrageenovora* has been proposed as playing a role in the degradation of carrageenan, as well as in arylsulfatase activity. Recently, we isolated a marine bacterium that produces arylsulfatase, which was confirmed by the hydrolysis of nitrophenyl sulfate and sulfate ester bonds in agar (Byun et al., 2004). The enzyme showed high specificity against agar, rather than other sulfated marine polysaccharides. Arylsulfatase hydrolyzed the sulfate ester bonds in agar without any glycosidase activity, which was demonstrated by the increased gelling strength of enzyme-treated agar (Kim et al., 2004; Byun et al., 2004). The enzyme enhances the gelling strength of

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agar by removing sulfate groups from agar; further reaction would convert agaropectin into agarose. This could replace the solvent fractionation step using solubility differentiation between agarose and agaropectin in agarose processing. The enzyme process would alleviate the use of organic solvents and reduce processing costs.

This paper establishes the conditions for inducing recombinant arylsulfatase from *P. carrageenovora* in *E. coli*, and the localization of the recombinant protein.

Materials and Methods

Bacterial strain, plasmid, and cell culture

The bacterial host strain used to express the recombinant arylsulfatase was *E. coli* BL21(DE3). The expression plasmid vector, pAST-A1, was previously constructed by inserting the ORF of arylsulfatase cloned from *P. carrageenovora* genomic DNA into the *E. coli* expression plasmid pET21a(+) (Novagen, Germany) (Lim et al., 2004). Plasmid pAST-A1 was introduced into *E. coli*, and the cells were grown on Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 50 μg per mL ampicillin for selecting transformants. Single colonies of *E. coli* cells with pAST-A1 were cultured on 2 mL LB medium supplemented with 50 mg/mL ampicillin in a shaking incubator at 37°C and 170 rpm overnight. The cultured cells were transferred to 200 mL of LB medium with 50 mg/mL ampicillin and cultured under the same conditions. For the expression of recombinant arylsulfatase, isopropyl-D-thiogalacto-pyranoside (IPTG, Sigma, St. Louis, MO) was added at concentrations between 0 and 5,000 μM at an OD_{600} of 0.8, and the cells were cultured for 6 to 14 hours more at 37°C. The grown cells were collected by centrifugation at 6,000×g for 20 min at 4°C.

Determination of enzyme activity

Arylsulfatase activity was determined by measuring the amount of p-nitrophenyl released from p-nitrophenyl sulfate (NPS). An assay mixture containing 1 mL of diluted enzyme solution and 250 μL of 25 mM NPS (pH 7.0) was incubated at 45°C for 30 min. The reaction was stopped by adding 1 mL of 0.5 M NaOH, and the p-nitrophenol produced was quantified spectrophotometrically at 410 nm. The sulfatase activity (U) was defined as the absorbance equivalent to 1 mole of p-nitrophenol produced per min per ml of enzyme solution under the experimental conditions. The specific activity was expressed as the absorbance equivalent of 1 mole p-nitrophenol produced by 1 mg of protein per min.

Osmotic disruption of *E. coli* cells

The cell pellets were then subjected to osmotic shock to release the recombinant arylsulfatase protein from the periplasmic space. The method was as follows, with a slight modification from the original procedure (Nossal and Heppel, 1966). The cell pellets were resuspended in 65 mL of 10 mM Tris-HCl buffer (pH 7.6) containing 30 mM NaCl, and were centrifuged at 2,500×*g* for 20 min at room temperature. The resulting pellets were resuspended in 35 ml of the same buffer, and centrifuged at 3,900×*g* for 20 min at room temperature. The supernatant was discarded, and the resulting cell pellets were resuspended in 10 ml of 33 mM Tris-HCl buffer (pH 7.6) with stirring. To this were added 10 mL of 40% sucrose/0.1 mM EDTA/33 mM Tris-HCl buffer (pH 7.6) while stirring rapidly, and the cell suspension was left to stir for 10 min at 25°C. The suspension was then centrifuged at 15,500×*g* for 20 min at 4°C. The resulting pellet was resuspended rapidly in 8 mL of ice-cold 0.5 mM $MgCl_2$, and was further centrifuged at 15,500×*g* for 20 min at 4°C. The resulting supernatant was saved as the periplasmic fraction and was used for subsequent purification procedures. The pellet was saved and resuspended in 8 mL of 10 mM Tris-HCl buffer (pH 7.6) as the cytoplasmic fraction.

Electrophoresis

SDS-PAGE was performed at room temperature using the method of Laemmli (1970) with 10% polyacrylamide gels containing 1% SDS. After electrophoresis, the SDS-polyacrylamide gel was stained with Coomassie Brilliant R.

Results and Discussion

Effect of IPTG concentration

The effect of IPTG concentration on arylsulfatase activity was determined at various concentrations of IPTG from 0-5,000 μM. As shown in Fig. 1, most of the arylsulfatase activity was found in the cell lysate until the addition of 10 μM IPTG. In the IPTG concentration range from 50 to 5,000 μM, activity was observed in both the culture supernatant and cell lysate. The secreted enzyme activity increased with the IPTG concentration. To quantify the enzyme activity in the intra- and extracellular fractions, enzyme expression was induced by 10 and 50 μM of IPTG. Ninety-five percent of the total activity was detected in the cell lysate after induction with 10 μM IPTG, while 43% of the total activity was detected in the

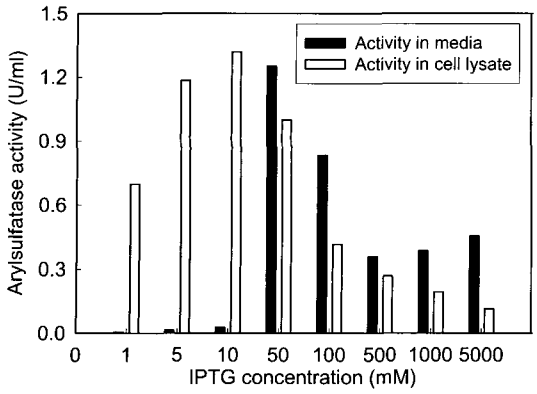

Fig. 1. Effect of IPTG concentration on the expression of arylsulfatase. Induction was performed with the addition of indicated concentration of IPTG at OD_{600}=0.8. Cells and media were harvested after 8 hr culture.

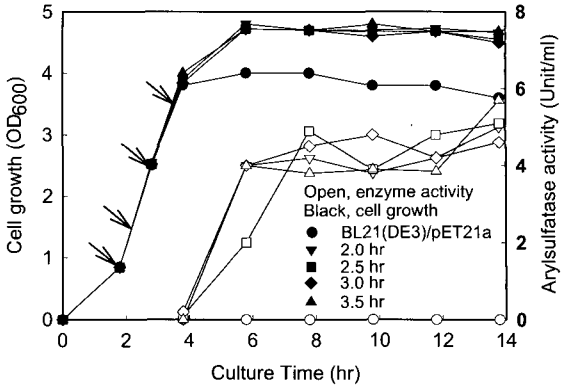

Fig. 2. Effect of induction time on the cell growth and arylsulfatase activity. Induction was performed with the addition of 10 μM of final IPTG concentration at indicated time and cells were harvested at indicated time.

Table 1. Comparison of cell growth and localization of arylsulfatase activity with the induction of 10 and 50 μM of final IPTG concentration

IPTG conc. (μM)	Cell growth (OD_{600})	Arylsulfatase activity (U/mL)	
		Supernatant	Cell lysates
10	3.46	0.10	1.94
50	2.06	1.04	0.80

cell lysate after induction with 50 μM IPTG (Table 1).

A similar result is often observed in the expression of heterologous genes in *E. coli*. In the expression of *Bacillus subtilis* pectate lyase in *E. coli*, the localization and secretion of the recombinant protein varied with the inducer concentration (Matsumoto et al., 2002). The concentration of IPTG also changed the expression levels of cyclodextrin glucanotransferase (Kwon et al., 2002) and the cholera toxin A2B subunit in *E. coli* (Lee et al., 2003).

Recombinant arylsulfatase in *E. coli* was maximally induced with intracellular protein with 10 μM IPTG. The expression of recombinant protein and cell growth were determined with various induction times using 10 μM IPTG.. As shown in Fig. 2, cell growth and arylsulfatase activity did not change with the induction time, and the level of arylsulfatase expres-sion was maintained at 4-5 U/mL after a 6-hr culture. The arylsulfatase activity in the flask culture was four times higher than that in the test-tube culture (data not shown).

Recombinant protein localization

Most of the activity was detected in the periplasmic space (Table 2). The osmotic disruption of *E. coli* cell membranes released the periplasmic contents, including most (81%) of the arylsulfatase activity.

Table 2. Localization of arylsulfatase activity in *E. coli* with the induction of 10 μM of final IPTG concentration

	Prontein conc. (mg/mL)	Activity (U)	Specific activity (U/mg)	Recovery (%)
Periplasm	0.96	0.51	0.53	81
Cytoplasm	7.75	1.43	0.18	19

The specific activity of the periplasmic fraction was four times higher than that of the cytoplasmic fraction, which is a good isolation step for arylsulfatase. The efficiency of periplasmic fractionation was also confirmed by analyzing the SDS-PAGE profile of the

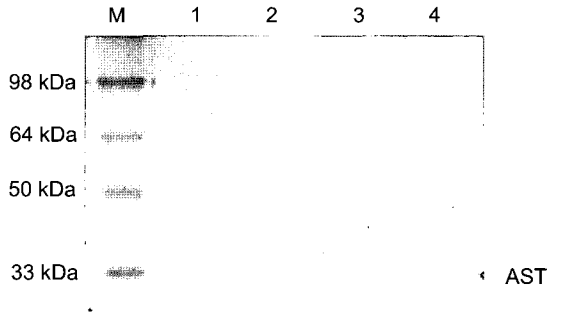

Fig. 3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of arylsulfatase expressed in *E. coli*. Proteins were separated on 10% SDS-PAGE gel and stained with Coomassie blue. M, molecular weight marker; lane 1, total cell lysate from *E. coli* BL21(DE3)/pET21(+); lane 2, total cell lysate from *E. coli* BL21(DE3)/pAST-A1; lane 3, periplasmic fraction from *E. coli* BL21(DE3)/pET21(+); lane 4; periplasmic fraction from *E. coli* BL21(DE3)/pAST-A1.

proteins present in each fraction. As shown in Fig. 3, the cytoplasmic fraction (lane 2) contained protein species different from those present in the periplasmic space (lane 4). Hence, the periplasmic fraction was suitable for the subsequent purification procedures.

About 80% of the arylsulfatase induced with 10 μM IPTG was located in the periplasmic space, which is consistent with previous reports (Fitzgerald and George, 1977; Barbeyron et al., 1995). The arylsulfatase from *P. carrageenovora* was mainly distributed in the periplasmic space (Barbeyron et al., 1995). When the transformed cells were fractionated into the periplasmic space and cytoplasm, activity was detected in both fractions. The localization of arylsulfatase in the periplasmic space should simplify the purification of the arylsulfatase.

Acknowledgments

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