The Journal of Microbiology, December 1999, p.200-205 Vol. 37, No. 4

Isolation of Novel *Pseudomonas diminuta* KAC-1 Strain Producing Glutaryl 7-Aminocephalosporanic Acid Acylase

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(Received October 25, 1999 / Accepted November 25, 1999)

7-Aminocephalosporanic acid (7-ACA) is the initial compound in preparation of cephalosporin antibiotics widely used in clinical treatment. Bacteria producing glutaryl 7-ACA acylase, which convert cephalosporin C to 7-ACA, has been screened in soil samples. A bacterial strain exhibiting high glutaryl 7-ACA acylase activity, designated KAC-1, was isolated and identified as a strain of *Pseudomonas diminuta* by characterizing its morphological and physiological properties. The screening procedures include culturing on enrichment media containing glutaric acid, glutamate, and glutaryl 7-aminocephalosporanic acid as selective carbon sources. To enhance enzyme production, optimal cultivation conditions were investigated. This strain grew optimally at pH 7 to 9 and in temperatures of 20 to 40°C, but acylase production was higher when the strain was grown at 25°C. Glutaric acid, glutamate and glucose also acted as inducers for acylase production. In a jar fermenter culture, *P. diminuta* KAC-1 produce acylase in a growth-associated manner. The substrate specificity of KAC-1 acylase by cell extract showed that this enzyme had specificity toward glutaryl 7-ACA, glutaryl 7-ADCA, but not cephalosporin C.

Key words: *Pseudomonas diminuta* KAC-1, glutaryl 7-aminocephalosporanic acid, cephalosporin C, glutaryl 7-aminocephalosporanic acid acylase, 7-aminocephalosporanic acid

7-ACA is an essential intermediate in the production of various clinically important semi-synthetic cephalosporin antibiotics. It has been prepared from cephalosporin C (CPC) by cleavage of the acyl side chain using chemical processes (7, 14) or enzymatic processes. Due to environmental and safety problems which occur in chemical processes, the enzymatic conversion method has been recently preferred for production of 7-ACA (17). CPC could be converted to 7-ACA by single-step or two-step enzyme processes with cephalosporin acylase which are classified into two types, namely CPC acylase and glutaryl 7-ACA acylase, according to their substrate preference. CPC acylase, capable of directly deacylating CPC, is required for an efficient and economic production of 7-ACA by the single-step process. CPC acylase was detected in species of *Pseudomonas* and filamentous fungi (15), but the enzymes characterized so far have low specific activity for CPC. The unusual nature of the D-aminoadipyl side chain of CPC may cause CPC to be less susceptible to enzyme hydrolysis to form 7-ACA. Glutaryl 7-ACA acylases have been shown to efficiently remove the acyl side chain of glutaryl 7-ACA to give 7-ACA. Since glutaryl 7-ACA can be easily obtained by oxidative deamination of CPC with chemical oxidation or by enzymatic reaction with D-amino acid oxidase (6), the glutaryl 7-ACA acylases have been regarded to be useful for production of 7-ACA from glutaryl 7-ACA by a two-step enzymatic process which seems to be preferable to the single-step process in terms of the maximun yield of 7-ACA. Glutaryl 7-ACA acylases have been purified and characterized from strains of *Bacillus* (2), and *Pseudomonas* sp. GK-16 (11), C427 (12), SE83 (13), A14, N176, V12 (1), BL072 (4, 5). A few enzymes can convert cephalosporin C and glutaryl 7-ACA to 7-ACA, but their activities for cephalosporin C did not affect the application of these processes. In spite of the difficulty in finding glutaryl 7-ACA acylase in nature, the research of this enzyme continues.

In this work, we have surveyed a new cephalosporin acylase-producing bacterium for the enzymatic manufacture of 7-ACA. The isolation and biochemical characterization of this strain are described, and its culture conditions were investigated for the acylase production.

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Materials and Methods

Chemicals

7-ACA was purchased from Sigma (USA). Glutaryl 7-amino cephalosporanic acid which was used as a substrate in the acylase reaction was kindly supplied by Chongkundang Co. (Korea). Glutaric acid and para-dimethylaminobenzaldehyde were obtained from Janssen (Belgium) and acetonitrile was from Budick and Jackson Lab. (USA). All other chemicals were of analytical grade.

Media and culture condition

Medium I consists of 1 g of yeast extract, 2 g of peptone, 30 g of monosodium glutamate (MSG), and 1 g of glutaric acid, and 1 g of glutaryl 7-ACA per liter of water. Medium II consists of 2 g of beef extract, 2 g of yeast extract, 5 g of peptone, 5 g of MSG, 1 g of glutaric acid per liter of water. Both were used for the isolation of bactcria producing glutaryl 7-ACA acylase. The pH of these media was adjusted to 7.2. Medium III is composed of 20 g of glucose, 3 g of beef extract, 5 g of peptone, 5 g of MSG, 1 g of glutaric acid per liter of water, and the pH was adjusted to 9.0. This medium was used to optimize the medium composition for the production of glutaryl 7-ACA acylase from *P. diminuta* KAC-1.

Screening for glutaryl 7-ACA acylase-producing microorganism

Soil collected from different sewage sludge, shallow streamlets, and mountains was enriched aerobically at 30°C on medium I. Once grown in the enrichment cultures, the supernatant was spread on agar plates of medium II. After being incubated for 1 to 3 days, colonies were isolated by transfer to the agar plates. A loopful of cells from an agar plate was suspended in 0.1 M Tris-HCl buffer (pH 8.0) and used for the glutaryl 7-ACA activity assay. To overcome the inhibition of glutaryl 7-ACA acylase by β-lactamase, potassium clavuranic acid was added to the reaction mixture as an inhibitor of β-lactamase.

Enzyme activity assays

Glutaryl 7-ACA acylase activity was assayed with the modified colorimetric method as described by Balasingham (3). A cell suspension (0.5 ml in 100 mM Tris-HCl buffer, pH 8.0) was incubated at 37°C in the presence of glutaryl 7-ACA (10 mM). The reaction was stopped by adding 3 ml of mixture of 20 % acetic acid and 0.05 M NaOH (2:1). After centrifugation, 0.5 ml para-dimethyaminobenzaldehyde (0.5% w/v in methanol) was added to the supernatant. The released 7-ACA was determined by measuring the absorbance at 410 nm. The procedure was originally developed for the determination of 6-aminopenicillanic acid and can also be applied for the detection of 7-ACA (16), the product of the enzymatic hydrolysis of glutaryl 7-ACA. In order to determine the quantities of glutaryl 7-ACA, 7-ACA and other analogous compounds, HPLC was performed. Reaction mixtures were injected on C18 reverse-phase HPLC column using a mobile phase of 20 mM ammonium acetate (pH 5.0) : ACN mixture (98:2). Compounds were detected at 250 nm. One unit of acylase activity was defined as the quantity of enzyme that produced 1 μmole of 7-ACA per min under the defined condition.

Identification of glutaryl 7-ACA acylase-producing microorganism

The isolate strain producing glutaryl 7-ACA acylase was identified from morphological and physiological properties according to Bergey's Manual of Systematic Bacteriology (9). The fatty acid compositions of the isolated strain were analyzed by the Microbial Identification System (MIDI Inc., USA).

Results and Discussion

Screening of acylase producing bacteria

Soil samples were collected from 200 districts in Korea and cultured on medium I broth. These enrichment cultures were plated on medium I agar plate and about 1000 bacterial strains were isolated in agar plate. These strains were incubated in medium II agar plate and glutaryl 7-ACA acylase activity of these microorganisms were confirmed by both the para-dimethylaminobenzaldehyde colorimetric assay and TLC assay methods. After screening by these assay methods, HPLC was performed to confirm the isolates showing glutaryl 7-ACA acylase activity. In many reports for screening of glutaryl 7-ACA acylase-producing bacteria, several derivatives of glutaryl 7-ACA were used for rapid process and circumventing substrate degradation by β-lactamase (4, 8, 18). But, these derivatives were not effective because the substrate specificity of glutaryl 7-ACA acylase is very narrow. Many isolates, which were active to them, had no activity toward glutaryl 7-ACA acylase. Thus we added glutaryl 7-ACA, cephalosporin C and 7-ACA to the screening medium for obtaining selectivity. Potassium clavuranic acid, an inhibitor of β-lactamase, was added to the reaction mixture for circumventing substrate degradation by β-lactamase of isolates. Three strains, which showed glutaryl 7-ACA acylase activity, were isolated by HPLC. Among these strains, one designated KAC-1 was finally selected as a strong producer of glutaryl 7-ACA acylase than other strains. The KAC-1 strain shows weak activity of β-lactamase.

Fig. 1. Scanning electron micrograph of *Pseudomonas* sp. KAC-1 strain.

Characterization and identification of KAC-1

The KAC-1 strain is a rod shaped, motile, non-sporulating, facultative aerobic, and Gram negative bacteria. As shown in Fig. 1, cell size was about 0.7~1.2 μm. The morphological, cultural, and physiological characteristics of strain KAC-1 are examined as shown in Table 1 and indicate that KAC-1 has characteristics similar to *Pseudomonas* sp. according to Bergey's Manual of Systematic Bacteriology, especially in sugar utilization. The fatty acid compositions of KAC-1 strain were examined using gas chromatography and compared with data of Microbial Identification System (Table 2). The fatty acids compositions of KAC-1 were similar to those of the *Pseudomonas* genus, especially *of P. diminuta.* From these results, the isolate was identified as *P. diminuta.*. In addition, KAC-1 strain was similar to *Pseudomonas* SY77-1 (16) and *Pseudomonas* BL072 (4) showing glutaryl 7-ACA acylase activity in many characteristics except their sugar utilization patterns. It was reported that crude cell extract of SY77-1 showed β-lactamase activity degrading almost reaction substrate and product for glutaryl 7-ACA acylase during enzyme reaction. While β-lactamase activity was detected in crude cell extract of the KAC-1 strain, the activity is expected to be lower than that of the SY77-1 strain.

Table 1. Morphological and biochemical properties of *Pseudomonas* sp. KAC-1

Characteristics	Results
Cell form	rods
Cell size (μm)	0.3~0.4 × 0.7~1.2
Motility	+
Gram staining	-
Spore formation	-
Anaerobic growth	-
Growth temperature	20~37°C
Growth pH	6~10
ONPG	-
ADH	+/-
LDC	-
OPC	-
Catalase	+
Oxidase	+
Indole production	-
Citrate	-
Urease	-
Lipase	-
Gelatin liquification	+
MR	+
VP	-
H_2S	+
β-lactamase	+
Starch hydrolysis	-
Casein hydrolysis	+
Litmus milk	-
Nitrate reduction	-
Acid forming from sugars including glucose, lactose, mannitol, inositol, sorbitol, rhamnose melibiose, arabinose, sucrose, maltose	-

+. Positive; -, Negative

Table 2. Fatty acid composition of cell membrane from *Pseudomonas* sp. KAC-1

Fatty acid	Composition (%)
11:0 iso	0.33
12:0 3OH	2.51
14:0	1.15
15:1 w8c	0.55
15:0	2.54
16:1 w7c	2.24
16:0	25.37
17:1 w8c	1.83
17:1 w6c	1.05
17:0	2.52
18:1 w7c/w9t/w12t	53.89
18:0	0.44
19:0 cyclo w8c	5.24
20:2 w6,9c	0.34

Characteristics of growth and enzyme production

To investigate cell growth and glutaryl 7-ACA acylase production of KAC-1, this strain was grown in a flask at various culture conditions on the basis of culture conditions of acylase-producing microorganisms such as *Pseudomonas* strain SY77-1, BL072, and other strains included in genus of *Pseudomonas*. Basic medium consisting of 0.3% beef extract and 0.5% peptone was used to produce the enzyme either with or without 1% organic compounds. As shown in Table 3, glutaric acid and MSG strongly induced glutaryl 7-ACA acylase production, and glucose increased acylase production and cell mass. As enzyme production of KAC-1 was highest in the presence of glutaric acid, the pro-

Table 3. Effects of organic compounds on glutaryl 7-ACA acylase production and cell growth

Organic compounds*	Relative productivity (%)	Cell growth (A600)
None	100.00	2.50
MSG	170.00	3.00
Glutaric acid	190.00	2.80
Casamino acid	60.00	4.40
Milk casein	55.00	5.50
Casein hydrolysate	52.00	4.90
Glucose	180.00	3.40

*Each of the organic compounds was added to medium at 1 % concentration.

cess was examined in medium supplemented with glutaric acid of various concentrations ranging from 0 to 2 %. The KAC-1 exhibited a 2 to 2.5-fold increase in glutaryl 7-ACA acylase by glutaric acid (Fig. 2A). It was reported that glutaric acid enhanced the productivity of glutaryl 7-ACA acylase in *Pseudomonas* strains except the BL072 strain (4, 8, 10, 11).

The effects of medium pH was also examined and optimized for the production of acylase. It was observed that final medium pH reached 8.8~9.2 after full growth in spite of the difference of initial pH of medium. However, the acylase productivity was dependent on the initial pH of medium. Unlike the strains SY77-1 and BL072, strain KAC-1 was identified to produce the highest level of acylase at pH 9 (Fig. 2B). Also, incubation temperature strongly influenced the productivity of acylase in KAC-1 (Fig. 2C). While cell growth of KAC-1 became maximum at 30°C, acylase production was maximum at 25°C. Enzyme production dramatically decreased over 30°C, though cell mass was maintained constantly. It is not clear why both medium pH and incubation temperature affect the productivity of glutaryl 7-ACA acylase. As cell mass was not influenced by these conditions, it was presumed that these factors influenced either the

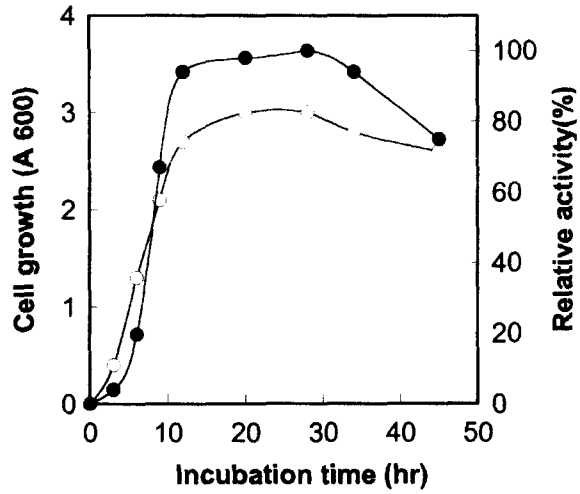

Fig. 3. Growth and glutaryl 7-ACA acylase production of *Pseudomonas* sp. KAC-1. KAC-1 strain was grown in medium III broth at 37°C with vigorous shaking. Glutaryl 7-ACA acylase activities were determined with the cell-free extract. Symbols; ○, cell growth; ●, relative activity (%) of the enzyme.

expression of the glutaryl 7-ACA acylase gene or post-translational processing for mature acylase.

The cellular growth and the production of acylase as a function of fermentation time are shown in Fig. 3 indicating acylase production is associated with cell growth. Maximum acylase activity was achieved at the end of the exponential phase and maintained by the stationary phase. The acylase activity was not found in the culture supernatant of actively growing cells, but cells were disrupted by autolysis and the enzyme was released in culture supernatant after 2 days of incubation.

Crude acylase was obtained from the KAC-1 cells both by sonication and French press steps. It was used to determine the substrate specificity of KAC-1 acylase with glutaryl 7-ACA and its related compounds (Table 4). This enzyme reacted with glutaryl 7-ACA

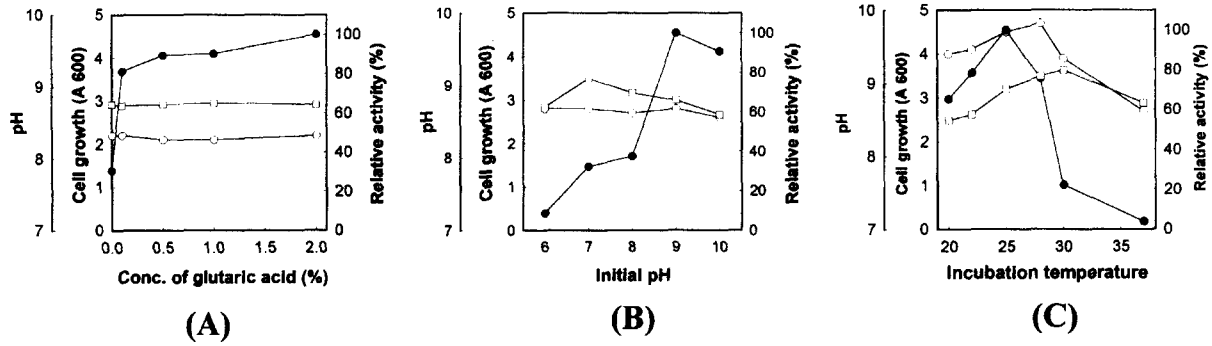

Fig. 2. Effects of environmental factors on glutaryl 7-ACA acylase production from *Pseudomonas* sp. KAC-1. (A), effects of glutaric acid concentrations; (B), effects of initial medium pH; (C), effects of incubation temperature. Symbols; □, final pH of culture broth; ○, cell growth; ●, relative activity (%) of the enzyme.

Table 4. Substrates specificity of KAC-1 glutaryl 7-ACA acylase

Substrates	Reaction*
Glutaryl 7-ACA	+
Glutaryl 7-ADCA	+
Cephalosporin C	-
Penicillin G, K	-
Ampicillin	-
Cefazoline	-
Arginine-pNA	+
Glycine-pNA	+
Leucine-pNA	+
Glutamine-pNA	+
Phenylalanine-pNA	-

*The reaction mixture contained 5 mM of respective substrate and 100 mM Tris · Hcl (pH 8.0).
+, Positive; -, Negative

and glutaryl 7-aminodeactyl cephalosporanic acid (glutaryl 7-ADCA) to produce 7-ACA and 7-ADCA, but not with cephalosporin C and other cephalosporin derivatives which contained a β-lactam ring. In addition, it was found that KAC-1 acylase could hydrolyze amino acid para-nitroanilide (pNA) compounds which were used as substrates of aminopeptidase. Arginine-, leucine-, glycine-, glutamine- and methionine- and phenylalanine-pNA were hydrolyzed by KAC-1 acylase, suggesting that KAC-1 acylase has aminopeptidase activity. Gamma-glutamyltransferase and transacylase activity of glutaryl 7-ACA acylase were found from some *Pseudomonas* (5, 10).

In many attempts to isolate bacteria producing glutaryl 7-ACA acylase, most of the strains were members of genus *Pseudomonas*. The enzymes from *Pseudomonas* strains can be divided into two groups as cephalosporin C acylase and glutaryl 7-ACA acylase according to its substrate specificity. KAC-1 acylase should be classified into the glutaryl 7-ACA acylase category with high similarity to SY77-1 and BL072 strains with regard to physiological characteristics and enzyme substrate specificity (4, 5, 16). In order to improve the KAC-1 acylase for enzymatic conversion of glutaryl 7-ACA into 7-ACA, future studies should focus on the characterization of acylase and high expression of the acylase gene.

References

1. **Aramori, I., M. Fukagawa, M. Tsumura, M. Iwami, H. Kojo, H. Ono, M. Kohsaka, Y. Ueda, M. Kohsaka, and H. Imanaka.** 1992. Comparative characterization of new glutaryl 7-ACA and cephalosporin C acylases. *J. Ferment. Bioeng.* **73,** 185-192.
2. **Aramori, I., M. Fukagawa, M. Tsumura, M. Iwami, H. Ono, H. Kojo, M. Kohsaka, Y. Udea, and H. Imanaka.** 1991. Cloning and Nucleotide sequence of a novel 7-(4-carboxybutanamido)cephalosporanic acid acylase gene of *Bacillus laterosoporus* and its expression in *Escherichia coli* and *Bacillus subtilis*. *J. Bacteriol.* **173,** 7848-7855.
3. **Balasingham K, D. Warburton, P. Dunnill, and M. Lilly.** 1972. The isolation and kinetics of penicillin amidase from *Escherichia coli*. *Biochim. Biophys. Acta.* **276,** 250-256.
4. **Binder, R., G.K. Numata, D.A. Lowe, T. Murakami, and J.L. Brown.** 1993. Isolation and characterization of a *Pseudomonas* strain producing glutaryl 7-aminocephalosporanic acid acylase. *Appl. Environ. Microbiol.* **59,** 3321-3326.
5. **Binder, R., J. Brown, and G. Romancik.** 1994. Biochemical characterization of a glutaryl-7-aminocephalosporanic acid acylase from *Pseudomonas* strain BL072. *Appl. Environ. Microbiol.* **60,** 1805-1809.
6. **Szwajcer-Dey, E., J.R. Miller, S. Kovacevic, and K. Mosbach.** 1990. Characterization of a D-amino acid oxidase with high activity against cephalosporin C from the yeast *Trigonopsis variabilis*. *Biochem. Int.* **20,** 1169-1178.
7. **Fechtig, B., H. Peter, H. Bickel, and E. Wischer.** 1986. Uber die herstellung von 7-aminocephalosporans aure. *Helv. Chim. Acta.* **51,** 1108-1119.
8. **Franzosi, G., E. Battistel, I. Gagliardi, and W. Van der Goes.** 1995. Screening and characterization of microorganisms with glutaryl-7ADCA acylase activity. *Appl. Microbiol. Biotechnol.* **43,** 508-513.
9. **Krieg, N.R. and J.G. Holt.** 1984. Bergey's manual of systematic bacteriology, vol. **1**, p. 141-199. The Williams & Wilkins Co., Baltimore, Maryland.
10. **Lee, Y.H., T.S. Chang, H.J. Liu, and W.S. Chu.** 1998. An acidic glutaryl-7-aminocephalosporanic acid acylase from *Pseudomonas nitroreducens*. *Biotech. Appl. Biochem.* **28,** 113-118.
11. **Ichikawa, S., Y. Murai, S. Yamamoto, Y. Shibuya, T. Fujii, K. Komatsu, and R. Kodaira.** 1981. The isolation and properties of *Pseudomonas* mutants with an enhanced productivity of 7β-(4-Carboxybutanamido)cephalosporanic acid acylase. *Agric. Biol. Chem.* **45,** 2225-2229.
12. **Ishii, Y., Y. Saito, T. Fujimura, T. Isoga, H. Kojo, M. Yamashita, M. Niwa, and M. Kohsaka.** 1994. A novel 7-β-(4-carboxybutanamido)-cephalosporanic acid acylase isolated from *Pseudomonas* strain C427 and its high-level production in *Escherichia coli*. *J. Ferment. Bioeng.* **77,** 591-597.
13. **Matsuda, A., K. Matsuyama, K. Yamamoto, S. Ichikawa, and K. Komatsu.** 1987. Cloning and characterization of the genes for two distinct cephalosporin acylase from a *Pseudomonas* strain. *J. Bacteriol.* **169,** 5815-5820.
14. **Morin, R.B., B.G. Jackson, E.H. Flynn, R.W. Roeske, and S.L. Andrews.** 1969. Chemistry of cephalosporin antibiotics. XIV. Reaction of cephalosporin C with nitrosyl chloride. *J. Amer. Chem. Soc.* **91,** 1396-1399.
15. **Reyes, F., M.J. Martinez, C. Alfonso, J.L. Copapatino, and J. Soliveri.** 1990. Cephalosporin C acylase in the autolysis of filamentous fungi. *J. Pharm. Pharmacol.* **42,** 128-131.
16. **Shibuya, Y., K. Matsumoto, and T. Fujii.** 1981. Isolation and properties of 7-(4-carboxybutanamido)cephalosporanic acid acylase-producing bacteria. *Agric. Biol. Chem.* **45,**

1561-1567.

17. **Walton, R.B.** 1964. Search for microorganisms producing cephalosporin C amidase. *Dev. Industrial. Microbiol.* **5,** 349-363.

18. **Zhang, Q.-J. W.-X. Xu, and L. Shi.** 1991. 2-Nitro-5-(6-bromohexanoylamino) benzoic acid test paper method for detecting microorganisms capable of producing cephalosporin acylases. *Anal. Biochem.* **196,** 201-206.