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Isolation and Characterization of Soil Strains Producing Glutaryl-7-Aminocephalosporanic Acid Acylase

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A search was undertaken to screen microorganisms that produce an enzyme capable of deacylating glutaryl-7-aminocephalosporanic acid to 7-aminocephalosporanic acid in soil samples. The screening was carried out by preparing enrichment cultures containing glutaryl-7ACA and cephalosporin C as selective carbon sources. A non-β-lactam model compound, glutaryl-p-nitroanilide, was synthesized as a substrate suitable for the rapid screening of microorganisms isolated from the enrichment cultures. Two isolates exhibiting acylase activity, designated BY7.4 and BY8.1, were identified as strains of *Pseudomonas* species. *Pseudomonas* BY8.1 showed higher acylase activity toward Gl-7ACA than *Pseudomonas* BY7.4. Environmental conditions for the optimal acylase activity of *Pseudomonas* BY8.1 were shown to be pH 9 and 30°C.

Key words: screening, 7-ACA, glutaryl-7ACA, cephalosporin acylase, bioconversion

INTRODUCTION

7-Aminocephalosporanic acid (7-ACA) is the starting material for the industrial production of most of semisynthetic cephalosporin antibiotics. It was originally hoped that an acylase as active as some of the penicillin acylases [1] would be found to convert cephalosporin C (CPC) directly to 7-ACA [2, 3]. However, acylases specific for the unusual D-aminoadipic acid side chain of cephalosporin C are rare [4]. Even the cephalosporin C acylases previously described [5, 6] generally have low activity and have not been proven industrially useful. Therefore, a two-step enzymatic deacylation of the cephalosporin C has been developed as an alternative to conventional chemical processes [7, 8]. This method has an advantage of avoiding most of problems associated with the classical chemical processes, since it does not require low temperature nor use of expensive and potentially polluting chemicals. The first step consists of the oxidative deamination of the cephalosporin C adipoyl side-chain by using D-amino acid oxidase (D-AAO) from yeasts [9,10]. In this reaction, a keto derivative, α-keto-adipyl-7-aminocephalosporanic acid, is spontaneously transformed to glutaryl-7-aminocephalosporanic acid (Gl-7ACA) in the presence of the hydrogen peroxide produced by D-AAO. The second step consists of the enzymatic hydrolysis of the side-chain of glutaryl-7ACA by a specific acylase obtained from bacteria [11, 12]. Usually, bacterial isolates with Gl-7ACA acylase activities also contain strong β-lactamase and esterase activities, which destroy Gl-7ACA before it can be converted to the desired beta-lactam nucleus. Model compounds that are closely related to Gl-7ACA are the most apt to be related as screening tools. In this report, we used glutaryl-p-nitroanilide as a non-β-lactam model compound for efficient screening of soil strains and described characterization of two strains showing Gl-7ACA acylase activity.

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MATERIALS AND METHODS

Chemicals

Yeast extract, casitone and nitrocefin were purchased from Difco. *p*-Nitroaniline, glutaric anhydride, corn steep liquor, dioxane were from Sigma. *p*-Dimethylaminobenzaldehyde and sodium glutamate were from Fluka. 7-ACA and cephalosprin C were obtained from Cheiljedang Company.

Preparations of GL-7ACA and Glutaryl-p-nitroanilide

The mixtures of 7-ACA and glutaric anhydride were stirred for 4 hours at room temperature and then extracted with ethylacetate as described previously [13]. The purity of glutaryl-7ACA was confirmed to be 98% by HPLC. For HPLC assays, a μ-Bondapak C18 (Waters) column was used and elution buffer was consisted of 0.567 g/l of Na_2HPO_4, 0.386 g/l of KH_2PO_4 and 3% methanol [5]. The detection wavelength was 254 nm. Typical retention times for 7-ACA and Gl-7ACA were 2.9 min and 2.6 min, respectively. Glutaryl-p-nitroanilide (GNA) was synthesized using p-nirtoaniline and glutaric anhydride as described previously [13].

Screening Microorganisms from Soil Samples

Various soil samples (0.1 g) taken from mountains in Kyongsan area were inoculated into test tubes (15 ml) containing isolation media (g/l): yeast extract, 1; NH_4Cl, 4; KCl, 0.5; K_2HPO_4, 1; $MgSO_4$, 0.5; $FeSO_4$, 0.05; $ZnSO_4$, 0.05; K_2CO_3, 0.05; GL-7ACA, 2; cephalosporin C, 1. After the samples were incubated for 10 days at 30°C, a portion of these cultures was spreaded on agar plates

containing glutaryl-p-nitroanilide (1 g/l). Colonies producing yellow colors on agar plates, due to the formation of p-nitroaniline, were randomly selected and incubated at 30°C in fermentation media containing (g/l) casitone, 20; yeast extract, 5; sodium glutamate, 5; corn steep liquor, 2; glutaric acid, 1. The fermentation cultures were harvested after 3 days and washed with 100 mM phosphate buffer (pH 7.5). The washed cells were permeabilized by three times of freeze-thaw treatments and then 5 mM Gl-7ACA dissolved in 100 mM phosphate buffer was added and incubated for 30 min at 30°C. Gl-7ACA acylase activity was measured by colorimetric method using p-dimethylaminobenzaldehyde [14]. Clavulanic acid was used to inhibit β-lactamase activity. β-lactamase activity was assayed by use of nitrocefin (Difco) and by measuring absorption at 260 nm [13].

Purification of Glutaryl-7ACA Acylase

Positive strains producing glutaryl-7ACA acylase were cultivated with fermentation media at 30°C in a 5 *l* fermenter. Cells were harvested after 3 days and then cell walls were disrupted by French press at 10,000 g. Ammonium sulfate was added to the cell extract to 30% (w/v) and then centrifuged at 12,000 rpm for 30 min. To the supernatant, more ammonium sulfate was added to be 70% (w/v). The precipitated proteins were dissolved in phosphate buffer (potassium phosphate buffer 100 mM pH 7.5, glycerol 10%, EDTA 2 mM, mercaptoethanol 5 mM) and dialyzed against the same buffer for 12 hours at 4°C. The dialyzed protein solution was concentrated by ultrafiltration and then applied to DEAE-Sephadex FF column equilibrated with 10 mM sodium pyrophosphate (pH 8.0). Proteins were eluted with an elution buffer (10 mM sodium pyrophosphate (pH 8.0), 2 mM EDTA, 5 mM mercaptoethanol, 10% glycerol) and a linear gradient of NaCl (500 mM). The acylase-positive fractions were collected and concentrated by ultrafiltration before use.

Electron Microscopy

Strains on agar plates were fixed with 2.5% glutaraldehyde-0.1M sodium phosphate buffer (pH7.4) and dehydrated with ethanol. The samples were critical point dried, coated with gold and then viewed with a Hitachi S-4100 scanning electron microscope.

RESULTS AND DISCUSSION

Initial attempts to isolate microorganisms containing Gl-7ACA acylase activity had been performed with 17 soil samples. Microorganisms in soil usually have weak cellular activities due to the lack of proper nutrients. To restore their cellular activities as well as to increase their cell numbers, each soil sample (0.1g) was put in sterile nutrient-rich, LB media (10 ml) and incubated overnight at 30°C. Then a portion of the mixed culture (10 μl) was inoculated into isolation media (10 ml) and incubated at 30°C. After 10 days of incubation, the cells in one milliliter of each mixed culture were collected and permeabilized by freezing-thawing treatment. The permeabilized cells were incubated with 2 mM glutaryl-7ACA for 30 min at 30°C and then reacted with p-dimethylbenzaldehyde (DBA). When measured the absorbance at 415 nm, the values of 5 soil samples (7, 8, 12, 16, 17) were higher than the other 12 samples as shown in Fig. 1. Thus, the five cultures, which increased the possibility to isolate microbial strains with Gl-7ACA acylase activities, were selected for further screening. Since the five samples contained mixed microbial strains, each sample was diluted to 105 times and streaked on agar plates containing glutaryl-p-nitroanilide dissolved in isolation media. The cleavage of glutaryl-p-nitroanilide resulted in producing yellow color due to the formation of p-nitroaniline. About 100 microbial colonies that had yellow diffusion halos were randomly selected from the agar plates. Each colony was tested for Gl-7ACA acylase activity. Two strains showed increased values of absorbance at 415 nm in both Gl-7ACA and glutaryl-p-nitroanilide assays as shown in Fig. 2. Accordingly strains 7.4 and 8.1 were isolated and cultivated in fermentation media.

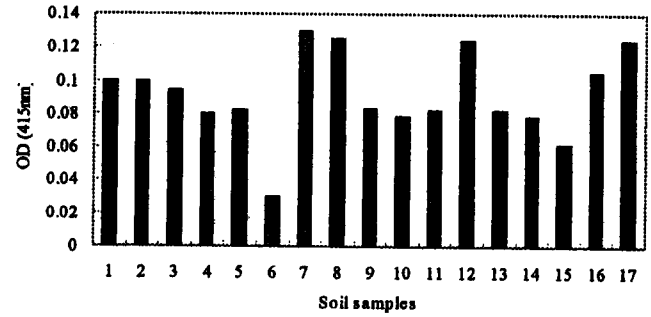

Fig. 1. Screening of acylase activities in mixed cultures obtained from soil samples.

In order to obtain the highest possible expression of the acylase activity, the fermentation conditions, including carbon sources and inducer concentration, were optimized for each strain. The optimal concentrations of glutamate and corn steep liquor were 0.5% (w/v) and 0.2% (w/v), respectively, for both strains (data not shown). By using the same fermentation medium, different glutaric acid concentrations were tested from 0 to 0.2 g/l as an acylase inducer. Strain 7.4 showed the maximal acylase activity with 0.1% (w/v) glutaric acid while strain 8.1 did not require any inducer to express the maximal activity (data not shown), indicating that it is a constitutive strain for acylase. The physiological properties of these two strains were listed in Table 1. Both strains were aerobic, gram-ne-

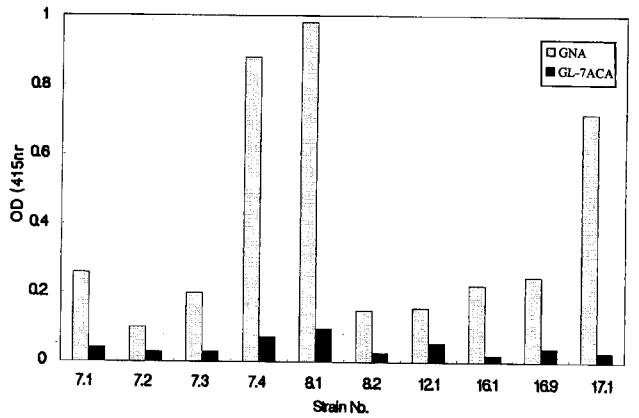

Fig. 2. Screening of acylase activities of microbial strains obtained from colonies on agar plates. Glutaryl-p-nitroanilide (GNA) and glutaryl-7ACA (Gl-7ACA) were used as substrates.

Table 1. Characterization of acylase positive strains

Characteristics	Strain 7.4	Strain 8.1
Reduction of nitrate to nitrite	+	+
Indole production	-	-
Acidfication (glucose)	+	+
Arginine dihydrolase activity	-	-
urease activity	-	+
β-Glucosidase (esculin) activity	+	+
Protease (gelatin) activity	+	+
β-Galactosidase activity	+	+
Utilization of Glucose	+	+
Arabinose	-	-
Mannose	+	+
Mannitol	+	+
N-acetyl-glucosamine	+	+
Maltose	+	+
Gluconate	+	+
Caprate	+	+
Adipate	-	-
Malate	+	+
Citrate	+	+
Phenyl-acetate	+	+

gative, non-spore forming, motile, short rod-shaped bacterium as shown in Fig. 3 and belonged to the genus of *Pseudomonas*. Thus, these strains were designated *Pseudomonas* BY7.4 and *Pseudomonas* BY8.1, respectively.

Pseudomonas BY8.1 showed higher acylase activity toward Gl-7ACA than *Pseudomonas* BY7.4 (Fig. 2). Thus, *Pseudomonas* BY8.1 was selected for further studies of its acylase activity. The crude cell extracts were prepared from 10 *l* of fermentation cultures and partially purified using DEAE-Sephadex ion-exchange column. The acylase active-fractions appeared before NaCl gradient elutions were loaded (data not shown). The active fractions were collected together and concentrated by ultrafiltration (Centriprep concentrators, Amicon). The optimal pH for this acylase activity was found to be pH 9.0, as shown in Fig. 4. This pH dependence was very similar to *Pseudomonas* N176 reported by Aramori *et al.* [6]. BY8.1 acylase activity was not measured in different buffers at the same pH but it could be more increased in different buffers since it was reported that N176 acylase activity assayed in the phosphate buffer and in the glycine buffer were 3.3 and 2 fold higher than those assayed in the Tris-HCl buffer, respectively [6]. The optimal temperature of BY8.1 acylase was 30°C (Fig. 5), which was different from that of N176 acylase, 45-50°C [6].

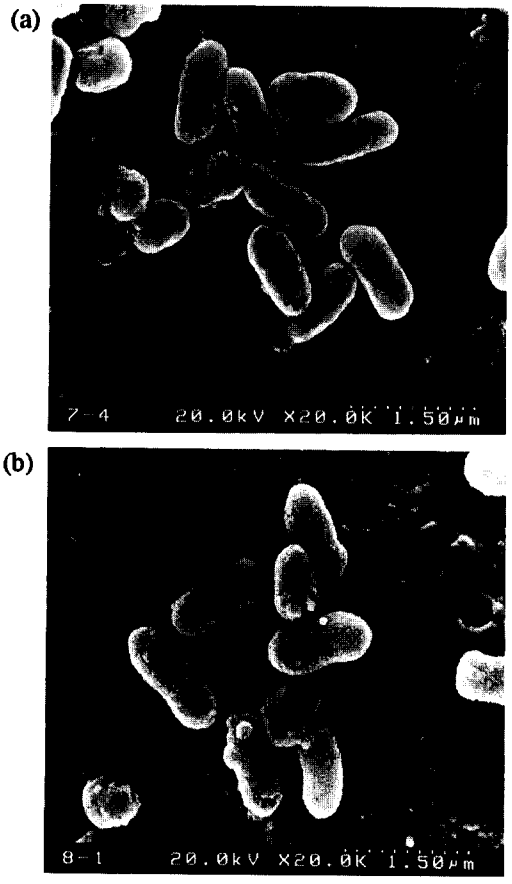

Fig. 3. Electron micrographs of soil strains (a) 7.4 and (b) 8.

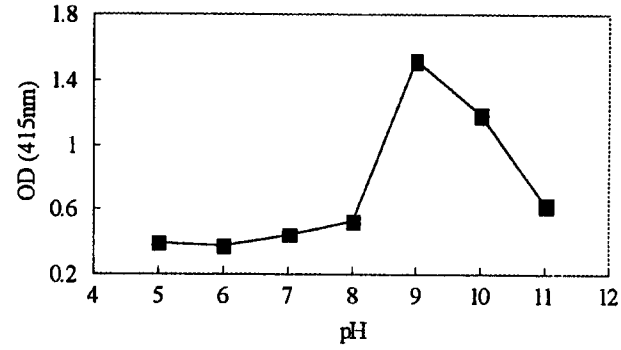

Fig. 4. pH-dependence of BY8.1 acylase activity.

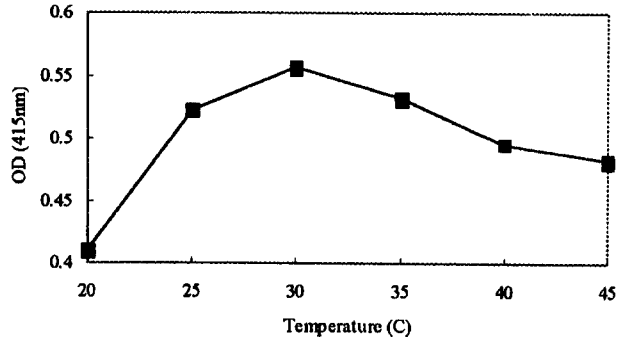

Fig. 5. Temperature-dependence of BY8.1 acylase activity.

The acylase active-fractions showed positive response to nitrocefin assay, indicating presence of β-lactamase. Although BY8.1 acylase activities were measured in the presence of β-lactamase inhibitor, clavulanic acid, it remains unclear how much the assays were influenced by the β-lactamase activity. Thus, further studies are needed with *Pseudomonas* BY8.1 acylase.

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REFERENCES

[1] Shewale, J. G. and V. K. Sudhakaran (1997) Penicillin V acylase: Its potential in the production of 6-aminopenicillanic acid. *Enzyme Microb. Technol.* 20: 402-410.

[2] Yamada, H., Y. Ishii, Y. Noguchi, T. Miura, T.

Mori, and Y. Saito (1996) Protein engineering of a cephalosporin C acylase. *Ann. N.Y. Acad. Sci.* 799: 74-81.

[3] Isogai, T., M. Fukagawa, I. Aramori, M. Iwami, H. Kojo, T. Ono, Y. Ueda, M. Kohsaka, and H. Imanaka (1991) Construction of a 7-aminocephalosporanic acid (7ACA) biosynthetic operon and direct production of 7ACA in *Acremonium chrysogenum*. *Biotechnology* 9: 188-91.

[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] Aramori, I., M. Fukagaw, M. Tsumura, M. Iwam, Y. Yokota, H. Kojo, M. Kohsaka, Y. Ueda, and H. Imanaka (1991) Isolation of soil strains producing new cephalosporin acylases. *J. Ferment. Bioeng.* 72: 227-231.

[6] Aramori, I., M. Fukaga, M. Tsumura, M. Iwami, H. Ono, Y. Ishitani, H. Kojo, M. Kohsaka, Y. Ueda, and H. Imanaka (1992) Comparative characterization of new glutaryl 7-ACA and cephalosporin C acylases. *J. Ferment. Bioeng.* 73: 185-92.

[7] Conion, H. D., J. Baqai, K. Baker, Y. Q. Shen, B. L. Wong, R. Noiles, and C. W. Rausch (1995) Two-step immobilized enzyme conversion of cephalosporin C to 7-aminocephalosporanic acid. *Biotechnol. Bioeng.* 46: 510-513.

[8] Alfani, F., M. Cantarella, N. Cutarella, A. Gallifuoco, P. Golini, and D. Bianchi (1997) Enzymatic conversion of cephalosporin C into glutaryl 7-aminocephalosporanic acid. A study in different reactor configurations. *Biotechnol. Lett.* 19: 175-78.

[9] Huber, F. M., J. T. Vicenzi, A. J. Tietz (1992) High yielding culture conditions for the biosynthesis of D-amino acid oxidase by *Trigonopsis variabilis*. *Biotechnol. Lett.* 14: 195-200.

[10] Pilone, M. S., L. Pollegioni, P. Casalin, B. Curti, S. Ronchi (1989) Properties of D-amino acid oxidase from *Rhodotorula gracilis*. *Eur. J. Biochem.* 180: 199-204.

[11] Aramori, I., M. Fukagawa, M. Tsumura, M. Iwami, H. Ono, H. Kojo, M. Kohsaka, Y. Ueda, and H. Imanaka (1991) Cloning and nucleotide sequencing of a novel 7β-(4-carboxybutanamido)cephalosporanic acid acylase gene of *Bacillus laterosporus* and its expression in *Escherichia coli* and *Bacillus subtilis*. *J. Bacteriol.* 173: 7848-55.

[12] Aramori, I., M. Fukagawa, M. Tsumura, M. Iwami, T. Isogai, H. Ono, Y. Ishitani, H. Kojo, M. Kohsaka, Y. Ueda, and H. Imanaka (1991) Cloning and nucleotide sequencing of new glutaryl 7-ACA and cephalosporin C acylase genes from *Pseudomonas* strains. *J. Ferment. Bioeng.* 72: 232-43.

[13] Franzosi, G., E. Battiste, I. Gagliardi, W. van der Goes (1995) Screening and characterization of microorganisms with glutaryl-7ADCA acylase activity. *Appl. Microbiol. Biotechnol.* 43: 508-513.

[14] Shewale, J. G., K. K. Kumar, and G. R. Ambekar (1987) Evaluation of determination of 6-aminopenicillanic acid by p-dimethyl aminobenzaldehyde. *Biotechnol. Techniq.* 1: 69-72.