The Isolation and Properties of *Pseudomonas* Mutants with an Enhanced Productivity of 7 β -(4-Carboxybutanamido)-cephalosporanic Acid Acylase[†]

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Isolation of mutants with an enhanced productivity of 7 β -(4-carboxybutanamido)-cephalosporanic acid acylase (penicillin amidohydrolase, EC 3.5.1.11) was attempted. A mutant, Ci-36, isolated by a method using glutarylanilide, produced approximately 5-times more acylase than did the parental strain. However, this acylase formation was still dependent on glutaric acid which was previously found to be essential in the case of the wild strain, *Pseudomonas* SY-77-1. The inducible-acylase formation was found to be firmly associated with the process of cell multiplication. Subsequently, a mutant, GK-16 was derived from Ci-36, which was shown to produce the acylase at maximum level without the addition of glutaric acid. The productivity of GK-16 was 2.4-times higher than that of Ci-36.

Much attention has been given to 7-ACA because of its usefulness as a starting material for the industrial synthesis of semisynthetic cephalosporins.

The compound has been chiefly prepared by removing chemically¹⁾ the side chain from the naturally occurring cephalosporin C. It was found originally by Shibuya *et al.* that GL-7ACA, obtained by deaminating chemically²⁾ or enzymatically^{3,15)} the side chain of cephalosporin C, is hydrolyzed readily into 7-ACA and glutaric acid by an enzyme of *Pseudomonas* SY-77-1^{4,5)} or *Pseudomonas putida* ATCC 950.^{4,5)} The enzyme named 7 β -(4-carboxybutanamido)cephalosporanic acid acylase (penicillin amidohydrolase, EC 3.5.1.11) was also shown in *Arthrobacter*.^{6,7)}

Wild strain, *Pseudomonas* SY-77-1, was also found to produce β -lactamase (EC 3.5.2.8) to a considerable extent in addition to GL-7ACA acylase. The use of the acylase preparation contaminated with β -lactamase activity was shown to be extremely unfavorable to the 7-ACA-producing process. Therefore, a mutant NS-2 lacking the β -lactamase activity was isolated from the wild strain, as a first step towards strain improvement.⁵⁾ However, the ability of NS-2 to produce the acylase is still dependent on glutaric acid and is too low to be applied to the industrial process. We have tried further to isolate mutants with a more enhanced productivity without the addition of glutaric acid.

This paper describes the isolation of mu-

[†] 7-Aminocephalosporanic Acid Production. Part II. For Part I, see ref. 5.

Abbreviations: 7-ACA, 7-aminocephalosporanic acid; GL-7ACA, 7 β -(4-carboxybutanamido)cephalosporanic acid (glutarylamiodecephalosporanic acid); 7-ADCA, 7-aminodeacetoxycephalosporanic acid; GL-7ADCA, 7 β -(4-carboxybutanamido)deacetoxycephalosporanic acid.

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tants and the properties of these mutants.

MATERIALS AND METHODS

Organisms. *Pseudomonas* NS-2⁵⁾ and its derivatives obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis were used throughout this work.

Media and culture conditions. The preculture was grown on bouillon agar slant at 32°C for two days. 100 ml of bouillon medium was placed in a 500-ml shaking flask and was sterilized at 120°C for 15 min. The flask was inoculated with a loopful of organism of the slant culture and incubated for 24 hours at 32°C on a rotary shaker operating at 220 rpm. Two production media were employed: Medium A containing milk casein *etc.*, as described previously,⁵⁾ was used for the strains, NS-2 and G-2. Medium B containing 3% Extract Bonito and 1% monosodium glutamate (pH 8.0) was used for the strains, Ci-36 and GK-16. A flask with 50 ml of the production medium was sterilized at 120°C for 15 min, inoculated with 2.5 ml of the seed culture and incubated at 25°C on a rotary shaker operating at 220 rpm.

Determination of cell yield. Cells were harvested from the culture by centrifugation at 13,000 × *g* for 5 min, washed with water and lyophilized. The lyophilized cells were weighed.

Enzyme assay. GL-7ACA acylase activity was determined by measuring 7-ADCA liberated from GL-7ADCA by washed cells suspended in 0.1 M phosphate buffer (pH 7.0), and 7-ADCA concentration was measured by the colorimetric method using *p*-dimethylaminobenzaldehyde according to Shibuya *et al.*⁵⁾

One unit of the acylase activity is defined as the amount to liberate 1 μmol of 7-ADCA per min at 37°C.

The acylase activity in intact cells was initially demonstrated to be equivalent to that in cell-free extracts when the high concentration of 7-ADCA (*e.g.*, 12 mM) was used as a substrate (*K_m* value of intact cells was about 1.8 mM.). Therefore, in this study, the acylase activity was assayed using intact cells in the presence of excess substrate (12 mM).

NTG mutagenesis. Cells growing exponentially in bouillon medium at 32°C were harvested and washed. Then the cells were suspended so as to give about 3×10^{10} cells per ml in water, and 1 ml of the cell suspension was added to 5 ml of 0.1 M phosphate buffer (pH 6.2) containing 50 μg of NTG per ml. After the suspension was incubated for 60 min at 32°C and diluted 10⁵ times with water, the cells were spread out on agar plate.

Preparation of glutarylanylilide. 30 g of aniline was dissolved in 300 ml of ethanol–water (1:1) and to the solution

12.3 g of glutaric anhydride was added. The mixture was stirred for 3 hours at room temperature, at pH 9.0 and concentrated *in vacuo* to remove ethanol. The concentrate was dissolved in water and the solution was extracted at pH 9.0 twice with benzene to remove excess aniline. The aqueous layer was then acidified to pH 5. The precipitate formed was collected and washed with acidic water. Recrystallization from methanol gave 10 g of white crystals (mp. 125 ~ 127°C).

Chemicals. 7-ADCA was supplied by Toyo Jozo Co., Ltd. GL-7ADCA was synthesized from 7-ADCA according to the method of the previous paper.⁵⁾ Bonito extract was purchased from Wako Pure Chemical Industries Ltd. Other materials used were the finest products available commercially.

RESULTS AND DISCUSSION

Isolation of mutants, G-2 and Ci-36, and properties

In order to isolate mutants having a high productivity of the acylase, a screening method using glutarylanylilide was designed.

In addition to GL-7ACA and GL-7ADCA, glutarylanylilide was found to be hydrolyzed into glutaric acid and aniline by the GL-7ACA acylase. Aniline was also shown to exert a strong-inhibitory effect on the growth of *Pseudomonas* NS-2. The minimum inhibitory concentration of aniline against strain NS-2 was 4 mg/ml, whereas those of glutarylanylilide and glutaric acid were 60 and 20 mg/ml, respectively.

It would seem highly likely, therefore, that a mutant with a higher productivity of acylase may fail to grow on agar plate containing glutarylanylilide because such a mutant can be expected to form a large amount of aniline from glutarylanylilide. According to this principle, we sought the mutants which are incapable of growing on medium A-agar plate containing 2 mg/ml of aniline and 20 mg/ml of glutarylanylilide and a mutant G-2 was isolated. Acylase synthesis of G-2 was about four times higher than that of NS-2 (Table I), but was still inducible and required glutaric acid for the maximum production of the acylase, as well as SY-77-1.⁵⁾

Moreover, the acylase synthesis of G-2 was unstable. Consequently, a stable mutant Ci-36

TABLE I. EFFECT OF GLUTARIC ACID ON THE ACYLASE FORMATION BY MUTANTS, G-2 AND NS-2

The mutants were grown in medium A at 25°C for 3 days.

Mutants	Acylase formed (units/ml)	
	With 1 mg/ml of glutaric acid	Without glutaric acid
NS-2	0.05	0.01
G-2	0.20	0.04

FIG. 1. Effect of Temperature on the Acylase Synthesis and Cell Yield.

Ci-36 was grown in medium B containing 0.1% of glutaric acid for 3 days. ●, acylase formed; ○, cell yield.

was selected from G-2.

The effect of temperature on the acylase synthesis by Ci-36

Since medium B was found to be suitable for the acylase synthesis by newly isolated Ci-36, the effects of temperature on the acylase synthesis and the bacterial growth were examined. As shown in Fig. 1, the optimal temperature for the acylase synthesis was 25°C, but the temperature range suitable for growth was from 25 to 35°C.

The effect of glutaric acid on the acylase synthesis

The effect of glutaric acid concentration on the acylase synthesis is presented in Fig. 2. Ci-36 produced only a small quantity of the acylase in the basal medium without glutaric

FIG. 2. Effect of Glutaric Acid Concentration on the Acylase Synthesis and Cell Yield.

Ci-36 was grown in medium B containing varying concentrations of glutaric acid for 3 days at 25°C. ○, acylase formed; ●, cell yield.

TABLE II. EFFECT OF COMPOUNDS ON THE ACYLASE SYNTHESIS

Ci-36 was grown in medium B containing 0.6 mg/ml of various compounds for 3 days at 25°C.

Compounds	Acylase formed (unit/ml)
Succinic acid	0.03
Glutaric acid	0.25
Adipic acid	0.02
Valeric acid	0.03
δ -Aminovaleric acid	0.02
Pentanediol	0.02
Glutaronitrile	0.03
Thiodiglycolic acid	0.38
Glutaric acid dimethylester	0.23
GL-7ACA	0.03

acid and the acylase synthesis was strongly accelerated by glutaric acid; The addition of 1 mg/ml of glutaric acid increased the enzyme level about five fold. When the concentration of glutaric acid was increased over 1.2 mg/ml, the acylase synthesis was gradually decreased.

The effect of glutaric acid analogs on the acylase synthesis was also examined. In addition to glutaric acid, glutaric acid dimethylester and thiodiglycolic acid were found to be an effective inducer, as shown in Table II.

The stimulatory effect of glutaric acid added to the culture in different phases of bacterial

FIG. 3. Effect of Glutaric Acid Added to the Culture in Different Phases of Growth.

Ci-36 was grown in medium B without inducer and 0.6 mg/ml of glutaric acid was added at different times (●, 0 hr; △, 18 hr; ▲, 26 hr; □, 35 hr; ■, 42 hr; ⊠, 52 hr; ○, no addition). A, acylase formation; B, cell yield.

growth on the acylase synthesis is presented in Fig. 3A. The strongest stimulatory effect was observed when the addition was made at zero and 18 hours. The acylase synthesis was substantially reduced in the cases of the addition in the later phases. The acylase activity in the culture to which glutaric acid was added during the stationary phase (52 hours) was very low and comparable to that in the culture without the addition of glutaric acid. The bacterial growth was observed for 10 to 50 hours as shown in Fig. 3B.

These observations would strongly suggest that the inducible synthesis of the acylase may be closely related to the process of cell multiplication and may occur only in the newly growing cells in the presence of glutaric acid, but not in the non-growing matured cells even on being exposed to the inducer. Szentirmai has made a similar observation in the case of *E. coli* penicillin acylase induced by phenylacetic acid.⁸⁾

On the other hand, the bacterial growth was inhibited a little by the addition of glutaric acid (Fig. 3B).

The isolation of a constitutive mutant

Since the growth of Ci-36 was slightly inhibited by the addition of glutaric acid and its acylase synthesis was also inhibited by the

FIG. 4. Effect of Glutaric Acid Concentration on the Acylase Synthesis and Cell Yield of GK-16.

GK-16 was grown under the same conditions as shown in Fig. 2. ○, acylase formed; ●, cell yield.

FIG. 5. Time Course of the Acylase Synthesis and Cell Yield of GK-16.

GK-16 was grown in medium B with 1 mg/ml of glutaric acid (○) or without inducer (●). Symbols: solid line, acylase formed; dotted line, cell yield.

addition of excess glutaric acid, it is to be expected that mutants producing the acylase constitutively without the addition of the inducer may form a more large amounts of the acylase. Therefore, a number of colonies of cells grown on agar plate containing medium B without glutaric acid were suspended in 0.1 M phosphate buffer (pH 7.0) and the acylase activities were examined. By this procedure, GK-16 was isolated as a constitutive mutant with improved productivity.

Properties of a mutant, GK-16

As shown in Figs. 4 and 5, the acylase synthesis and the cell yield of the mutant were confirmed to be independent of the glutaric

acid concentrations. The mutant was also found to produce 2.4 fold more acylase than did the parental strain, Ci-36, because the specific activities of both strains, GK-16 and Ci-36, were calculated to be 0.12 and 0.05 unit per mg dry cells from Figs. 4 and 2, respectively.

Since penicillin acylase was first found by Sakaguchi and Murao,¹⁰⁾ many penicillin acylases and cephalosporin acylases have been reported.^{9,11~13)} However, there has been no definite information on the regulation of enzyme synthesis or the physiological role including intracellular location, although the properties and the substrate specificity of these acylases are well-known.

Recently, however, two new-types of acylase were found. One is GL-7ACA acylase described in this paper and the other is the acylase hydrolyzing cephalosporin C into 7-ACA and α -aminoadipic acid.¹⁴⁾ Both enzymes are different from conventional penicillin and cephalosporin acylases in the substrate specificity. Therefore, further studies concerning GL-7ACA acylase are required.

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