Biochemical Characterization of a Glutaryl-7-Aminocephalosporanic Acid Acylase from *Pseudomonas* Strain BL072

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Received 7 January 1994/Accepted 2 April 1994

*Pseudomonas* strain BL072 produces an acylase enzyme active in hydrolyzing glutaryl-7-aminocephalosporanic acid to 7-aminocephalosporanic acid. This acylase was purified by column chromatography and gel electrophoresis. The native acylase was composed of two subunits of approximately 65 and 24 kDa, though some heterogeneity was seen in both the native acylase and its small subunit. The isoelectric point of the acylase is approximately 8.5, and it has a $K_m$ of 1.6 mM for glutaryl desacetoxycephalosporanic acid. The acylase hydrolyzes the desacetoxy and desacetyl derivatives of glutaryl-7-aminocephalosporanic acid at rates similar to that of glutaryl-7-aminocephalosporanic acid. Cephalosporin C was hydrolyzed at a reduced rate. The pH optimum was found to be 8.0, and an activation energy of 9 kcal/mol (ca. 38 kJ/mol) was observed. The acylase has transacylase activity 10 times that of its hydrolytic activity. Eupergit C-immobilized acylase had a half-life of greater than 400 h.

The β-lactam nucleus 7-aminocephalosporanic acid (7-ACA) is a key intermediate used in making semisynthetic cephalosporins from cephalosporin C. The chemical deacylation of cephalosporin C to make 7-ACA is a complex process utilizing expensive, toxic reagents and generating effluent with treatment problems. A much simpler method of making 7-ACA is to use an enzyme capable of directly deacylating cephalosporin C. However, few enzymes capable of this direct deacylation have been discovered, probably because of the unusual nature of the β-aminoacyl side chain of cephalosporin C. Acylases reported in the literature that can carry out this direct reaction have very low specific activities and have not yet proven economical to use industrially (2, 3, 10, 12, 15). A second enzymatic route first generates glutaryl-7-ACA [β-(4-carboxybutanamido)-cephalosporanic acid; GI-ACA] from cephalosporin C either chemically (20) or enzymatically (4, 11, 19, 21, 22) and then deacylates the GI-ACA with an acylase. Enzymes related to penicillin amidohydrolase (EC 3.5.1.1) are reported to have this acylase activity (1, 13, 14).

We have found such an acylase in a member of the *Pseudomonas diminuta* family, strain BL072. The microbiology of this strain was described in a previous paper (6). Here we describe the purification of the acylase, its physical and biochemical characterization, and its use in an immobilized form to convert GI-ACA to 7-ACA.

**MATERIALS AND METHODS**

**Chemicals.** Cephalosporin C, 7-ACA, and the desacetoxy derivative of 7-ACA (7-ADCA) were obtained from Bristol-Myers Squibb bulk manufacturing inventory. Glutaryl derivatives of the cephalosporin nuclei were prepared by acylation with glutaric anhydride followed by subsequent recrystallization (17). Desacetyl derivatives of cephalosporin C, glutaryl-ACA, and 7-ACA were prepared from the acetylated compounds by reaction with a *Rhodosporidium* esterase (18).

**Organism and growth conditions.** Strain BL072 was grown in batch culture in 300-liter fermentors as described in a previous paper (14). The medium contained 3% casein, 0.5% yeast extract, and 0.01% polypropylene glycol at pH 7.3. The cell mass was grown at 28°C for 40 h and harvested with a centrifuge (Westfalia CSA-8; 4 liters/min, 12,000 × g).

**Assays.** Acylase was assayed by mixing 0.5-ml samples containing 0.02 to 0.10 IU of acylase activity with 0.5 ml of 2% GI-ADCA (or another substrate) in 0.3 M Tris (pH 8.0) buffer. Mixture were agitated at 37°C for 30 min. Reactions were stopped by the addition of 4 ml of 1:1 50 mM KHPO$_4$-acetonitrile and centrifuged to give a clear supernatant.

Two microliters of the reaction supernatants was injected onto a 5-μm C$_18$ reverse-phase high-performance liquid chromatography (HPLC) column (4.2 by 50 mm) which had a mobile phase of 10 mM octyl sulfoxic acid, 10% methanol, and 0.1% phosphoric acid (final pH 2.5). The flow rate was 3 ml/min. Typical retention volumes for GI-ADCA and 7-ADCA were 1.8 and 4.2 ml, respectively. The void volume was 0.8 ml. Product formation was measured as micromoles per minute per millilitre of sample (international units per milliliter).

The acyltransferase reactions were run in a similar manner, except reaction mixtures contained 1% GI-ADCA, 1% amine acceptor, and 150 mM Tris (pH 8.0) buffer. Product formation was monitored by HPLC using a 5-μm C$_18$ column (4.2 by 100 mm) and a mobile phase consisting of 0.1% phosphoric acid with a 5-min gradient of 0 to 20% acetonitrile. The flow rate was 2.5 ml/min. Typical elution volumes for desacetyl-7-ACA, desacetyl cephalosporin C, 7-ADCA, 7-ACA, cephalosporin C, glutaryl-desacetyl-7-ACA, GI-ADCA, and GI-ACA were 0.78, 1.35, 2.15, 2.95, 5.2, 6.2, 8.5, and 9.2 ml, respectively.

Protein was assayed by the Coomassie blue G-250 binding assay of Bradford (7) with bovine serum albumin as the calibration standard. The salt concentration was measured with a conductivity probe.

**Purification of BL072 acylase.** A BL072 cell concentrate (6 liters, 500 IU, 1.4 kg of cells [wet weight], pH 8) was slurried in water containing 50 mg each of lysozyme and DNase, and the cells were broken with a Gaulin homogenizer. Ammonium sulfate was added to a final concentration of 2%, and the slurry was heated at 50°C for 30 min. The slurry was cooled (20°C), polyethyleneimine was added at pH 8.0 to a final concentration of 0.75% to flocculate the debris, and the mixture was centrifuged at 8,000 × g for 10 min. The supernatant was dialyzed on a hollow fiber unit (DC-10; Amicon) to a
TABLE 1. Summary of a stepwise purification process of BL072 acylase

<table>
<thead>
<tr>
<th>Stage</th>
<th>Yield (IU)</th>
<th>Sp act (IU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>390</td>
<td>ND*</td>
</tr>
<tr>
<td>Dialyrate</td>
<td>240</td>
<td>0.08</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>128</td>
<td>0.41</td>
</tr>
<tr>
<td>HTP hydroxyapatite</td>
<td>82</td>
<td>0.91</td>
</tr>
<tr>
<td>SP-Trisacryl</td>
<td>25</td>
<td>1.8</td>
</tr>
<tr>
<td>S-300 gel</td>
<td>24</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Activity and protein assays were done as described in Materials and Methods.

 conductivity equivalent to 40 mM NaCl. The pH was adjusted to 9.0, and 3 liters was loaded onto a Q-Sepharose column (4.5-cm diameter, 300 ml). After loading, the acylase was eluted with 600 ml of 20 mM Tris-Cl (pH 9.0) buffer containing a 0 to 0.6 M NaCl linear salt gradient.

The eluate fractions containing acylase activity were pooled and dialyzed against 10 mM potassium phosphate (pH 7.0), and the 100-ml pool was loaded onto a 4.5-cm diameter, 300-ml column of hydroxyapatite (Bio-Rad HTP). The acylase was eluted with a 400-ml linear phosphate gradient (0.01 to 0.6 M, pH 7.1). The fractions containing acylase were pooled and dialyzed against 20 mM sodium maleate –3 mM sodium azide (pH 5.8).

The dialysate (50 ml) was loaded onto an SP-Trisacryl (IBF Biotechnics) column (1-cm diameter, 30 ml) and eluted with 60 ml of a 20 mM maleate (pH 5.8)–salts (0 to 1 M NaCl) gradient. The peak fractions were concentrated to 2 ml with an Amicon ultrafiltration cell with a PM-10 membrane and loaded onto an S-300 column (2-cm diameter, 550 ml) containing 0.4 M NaCl and 0.1 M potassium phosphate (pH 7.2). Eluate fractions were concentrated 10-fold to 0.3 ml by ultrafiltration. Bovine serum albumin and immunoglobulin were used to calibrate the S-300 elution volume with respect to molecular weights.

**Electrophoresis.** Native polyacrylamide slab gel electrophoresis was run according to the method of Davis (8) using a 7% running gel and a 4% stacking gel. Fractions eluting from the S-300 column were mixed with glycerol and tracking dye and loaded in duplicate sections of the gel. The gel (250 by 150 by 1 mm) was run at 25 mA for 6 h. Half the gel was stained with Coomassie blue, and the duplicate half was sliced and electroeluted in small dialysis bags. The native eluates were concentrated with Centricon (Amicon) ultrafiltration membranes before assay and reloading onto gels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were run according to the method of Weber and Osborn (25). Molecular weight standards used were bovine serum albumin, ovalbumin, and myoglobin.

Isoelectric focusing gels were run by using the Phast system developed by Pharmacia. Calibration was done with the high-pI standard kit from Pharmacia.

**Im mobilization of acylase and use in conversion.** A crude cell extract of BL072 (11 IU/ml, 42 g of protein per ml, 160 ml in 1 M phosphate buffer [pH 7.4] and 3 mM sodium azide) was added to 40 g of dry Eupergit C (Rohm Pharma), and the damp mixture was incubated at 22°C for 3 days and washed according to the protocol in reference 9. The yield was 124 g of damp beads assaying at 8.7 IU/g at 37°C, giving a 63% activity yield. A 20-g portion of this material was placed into a packed bed reactor in line with a peristaltic pump and a reservoir for pH control.

Glutaryl-desacetyl-7-ACA for conversion was prepared by ultrafiltration and diluting cephalosporin C fermentation broth (Bristol-Myers Squibb), oxidizing the cephalosporin with Trigonopsis ν-amino acid oxidase (4, 11, 19, 21, 22), and removing the ester with esterase from Rhodospirillum cells (18). After filtration, 1 liter of crude solution containing 10 g of glutaryl-desacetyl-7-ACA per liter was recirculated through the column. The pH was controlled at 8.0 by titration with 1 N NaOH. The initial titration rate over the first 2 h was used to calculate column activity. The reaction was run at 22°C for 20 h, after which the system was recharged with fresh glutaryl-desacetyl-7-ACA.

**RESULTS AND DISCUSSION**

**Purification and physical characterization of the acylase.** A summary of the purification of the acylase is shown in Table 1. Cells of BL072 were homogenized, and the cell extract was heat treated at 50°C in the presence of 2% ammonium sulfate, as described in Materials and Methods. The ammonium sulfate was required for preservation of acylase activity at 50°C; nearly complete inactivation was seen without ammonium sulfate at 50°C. The heat treatment, though causing some acylase inactivation, significantly lowered contaminating esterase and β-lactamase activities. The mixture was centrifuged, and the supernatant was dialyzed to lower the salt concentration sixfold. The dialysate was raised to pH 9, loaded onto a Q-Sepharose column, and eluted with a salt gradient (Table 1). The fractions containing the amidase activity eluted at approximately 180 mM NaCl and showed a 5.5-fold increase in specific activity; these fractions were pooled and dialyzed. The second dialysate was loaded onto a hydroxyapatite column and eluted with a phosphate gradient, yielding another 2.2-fold purification. The peak fractions, eluting at approximately 100
TABLE 2. Relative rates of BL072 acylase activity against cephem substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate</th>
</tr>
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<tbody>
<tr>
<td>Gl-ACA</td>
<td>100</td>
</tr>
<tr>
<td>Glutaryl-desacetoxy-7-ACA</td>
<td>100</td>
</tr>
<tr>
<td>Glutaryl-desacyl-7-ACA</td>
<td>98</td>
</tr>
<tr>
<td>α-Ketoacyl-7-ACA</td>
<td>27</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>1</td>
</tr>
<tr>
<td>Desacetyl-cephalosporin C</td>
<td>1</td>
</tr>
</tbody>
</table>

*The reaction mixtures contained the substrate at 10 g/liter, acylase, and 100 mM Tris at pH 8.0. After reaction, the mixtures were assayed by HPLC as described in Materials and Methods.

mM phosphate, were pooled and dialyzed. This third dialysate was loaded onto an SP-Trisacryl column at pH 5.8 and eluted with a salt step gradient, although about half the activity did not adsorb to the column. The acylase activity eluted in 100 mM NaCl with a specific activity of 1.8 IU/mg, yielding another twofold purification. Finally, the concentrated pool was loaded on an S-300 gel sizing column. The acylase activity eluted in fractions corresponding to a molecular mass of approximately 80 to 90 kDa. The peak fractions had a specific activity of 2.7 IU/mg of protein. Yields were low throughout this purification because only peak fractions were carried through to the next stage.

Further purification was done by native gel electrophoresis. The purified acylase from the S-300 column migrated as two major and two minor bands on a native gel (Fig. 1A). The native acylase migrated only a short distance into the gel compared with bovine serum albumin (lane 3), reflecting the low charge of this acylase at pH 9. All four bands expressed GI-ACA acylase activity approximately in proportion to their protein concentration, with the upper and lower major bands (bands 2 and 3 in Fig. 1A) having specific activities of 2.4 and 2.9 IU/mg of protein, respectively. When the native bands were isolated and eluted from the gel and reapplied to a native gel, each individual band migrated as it did in the previous gel and did not redistribute into other bands. The two minor bands (bands 1 and 4) were not analyzed further. The two major native gel bands gave a two-subunit banding pattern when the eluted protein was run on an SDS-PAGE gel (Fig. 1B). The large subunit corresponded to a molecular mass of 65 kDa on SDS-PAGE gels, but the small subunit showed heterogeneity. The predominant small subunit from native band 2 had a molecular mass of 24 kDa (lanes 1 and 3), and the predominant small subunit from band 3 had a molecular mass of 23 kDa (lane 4). Also apparent in Fig. 1B is that the treatment given the acylase influences the banding pattern. Repeated freeze-thaw cycles caused a degradation of the large subunit to a lower molecular weight. Assuming that BL072 acylase consists of these two subunits, the purity of the peak S-300 fractions was estimated from SDS-PAGE gels to be greater than 97%, and the corresponding kcat (turnover number) is 4 s⁻¹.

The isoelectric point of the protein was determined with a Phast isoelectric focusing gel. The two major bands had pI values of 8.6 and 8.4 (data not shown). This slightly basic pI is a likely cause of the poor adsorption of the acylase to Q-Sepharose at pH values below 9.0 and the slow migration of the acylase on native gels.

Assuming that the BL072 procyclase is processed into two subunits in a manner similar to that of penicillin G amidohydrolase (5, 16) and aoyl (14), we believe that the microheterogeneity seen in the banding pattern on gels stems from different extents of proteolytic hydrolysis as the two subunits are formed from the precursor. This assumption is supported by sequence homologies (which will be presented in a later paper) between the BL072 acylase and these other two acylases.

Biochemical characterization of BL072 acylase. The acylase from BL072 does not display a strong selectivity between the β-lactam moieties to which the glutaryl side chain is attached. Table 2 shows that GI-ACA, glutaryl-desacetyl-7-ACA, and GI-ADCA are equally good substrates for the hydrolytic reaction, with relative rates within 5% of each other. GI-ADCA was used as a substrate in most acylase tests because of its relatively high stability (14).

FIG. 2. Temperature dependence of acylase velocity. Equal amounts of acylase were incubated in 1% GI-ADCA as described in Materials and Methods at four temperatures. Product formation was monitored over a 2-h time course. Open circles, 3°C; closed circles, 15°C; open triangles, 23°C; closed triangles, 37°C. (Inset) Arrhenius replot of the data showing reciprocal degrees Kelvin versus the natural log of the relative rates. The slope of the replot line yields an activation energy of 9.2 kcal/mol (ca. 38 kJ/mol).
In contrast, the substitution of the glutaryl side chain had a marked influence on the acylase activity. Ketaoidipyl-desacetyl-7-ACA was one-fourth as active as a substrate, and cephalosporin C was cleaved at a rate only 1% as great as that with Gl-ACA. No activity was detected against ketaoidipyl-7-ACA; this lack of activity might be due to its instability under assay conditions.

By using S-300-purified acylase, the $K_a$ for the hydrolysis reaction was found to be 0.54 mg/ml (1.6 mM) for Gl-ADCA (data not shown). The temperature dependency of the reaction was determined at temperatures between 3 and 37°C, and an activation energy of 9 kcal/mol (ca. 38 kJ/mol) was found (Fig. 2). This corresponds to a 1.7-fold increase in rate from a 10°C temperature rise within the range tested. The acylase is inactivated above 50°C.

The acylase was found to be stable between pHs 5 and 10, and optimum activity was found near pH 8. Reactions were run at a pH of 8.0 to minimize decomposition of the β-lactam molecule at higher pH values.

The equilibria achieved in Gl-ADCA conversions were also influenced by pH and other factors acting through mass-action effects. For example, with 10 g of Gl-ADCA per liter as a substrate at pH 6, the hydrolysis reaction reached an apparent equilibrium at approximately 70% conversion, whereas at pH 7 and 8, the apparent equilibria were at 90 and 95% conversion. Glutaric acid at 50 g/liter could inhibit the hydrolysis rate of 1 g of Gl-ADCA per liter by 50%. The back reaction (condensation) could be observed between 7-ACA and glutaric acid, particularly at pH values between 5 and 7.

Of particular interest was the effect of amines similar to the product cephalosporin nucleus upon the hydrolysis reaction. Apparent stimulation of Gl-ADCA hydrolysis by 7-ACA was found to be due to the enzyme working as an acyltransferase, transferring the glutaryl moiety from 7-ADCA to 7-ACA (Fig. 3). The initial rate of 7-ADCA formation is increased 10-fold by the addition of 7-ACA. When the enzyme is working as an acyltransferase, the reaction rate could also be stimulated 2- to 10-fold with such amine acceptors, including glycerylglucine and glutathione (Table 3). Reaction mixtures with an amine acceptor rapidly approach an equilibrium with the glutaryl moiety distributed between the available amine acceptors (Fig. 3).

Once a transitory equilibrium was achieved, the slower hydrolysis reaction (with water acting as the glutaryl acceptor) became the dominant net reaction. Similar acyltransferases act as transpeptidases involved in glutathione metabolism (23, 24).

**Immobilization of the acylase.** A crude preparation of acylase similar to the material loaded onto the Q-Sepharose column mentioned above (11 IU/ml, 42 g of protein per liter) was immobilized on Eupergit C resin. The washed resin expressed 63% of the activity in the loaded solution and had an activity of 9 IU/g (wet weight) at 37°C. A column was packed with this resin and then used to convert crude glutaryl-desacetyl-7-ACA to desacetyl-7-ACA. This substrate came from an active cephalosporin C fermentation broth that was ultrafiltered, enzymatically decacylated, and oxidized to glutaryl-desacetyl-7-ACA. Glutaryl-desacetyl-7-ACA was used because it had greater stability than Gl-ACA during a prolonged reaction. The pH of the filtrate was brought up to 8.0 before the reaction, and NaOH was used to control the pH at 8.0 as the solution was recirculated through the column. The NaOH titration rate over the first 2 h of the reaction was used to calculate the expressed activity of the column reactor. Figure 4 shows that the expressed activity dropped from an

![Graph](image)
initial velocity of 173 μmol/min in the first reaction to approximately 100 μmol/min after 20 20-h conversion cycles. Further examination of the resin in the packed column revealed that much of the lost activity could be restored by washing away a fine precipitate that collected in the column reactor during the course of the reactions. This precipitate slowly crystallized from the crude glutaryl-desacetyl-ACA solution as the reaction progressed at pH 8.0.

The acylase from BL072 could be a useful catalyst for the enzymatic conversion of Glu-ACA to 7-ACA. Its kinetic constants allow it to work in a range of substrate concentrations and physical conditions suitable for an aqueous β-lactam in an industrial process. The final evaluation of its utility will be the economic feasibility and environmental advantages of this enzymatic process as compared with the traditional chemical splitting manufacturing process.

ACKNOWLEDGMENTS
We thank K. Numata and M. Murakami for their discovery of and initial work on strain BL072. The excellent analytical work of B. Compton, M. Lewis, and G. Countryman was essential for the completion of this research. We also thank E. Walsh, D. A. Lowe, R. P. Elander, and J. J. Usher for their helpful suggestions and discussions.

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