Physical Properties and Kinetic Behavior of a Cephalosporin Acetylesterase Produced by Bacillus subtilis

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An esterase that deacetylates cephalosporins was recovered from the supernatant of a Bacillus subtilis culture. It was partially purified by ammonium sulfate fractionation and ultrafiltration. The enzyme had a temperature optimum between 40 and 50°C and a pH optimum of 7.0. The molecular weight was estimated by gel filtration to be 190,000. The enzyme was very stable and retained greater than 80% of its activity after storage in solution at 25°C for 1 month. The esterase exhibited Michaelis-Menten kinetics with the substrates 7-aminocephalosporanic acid (7-ACA) and 7-(thiophene-2-acetamido)cephalosporanic acid (cephalothin); the $K_m$ values were $2.8 \times 10^{-3}$ and $8.3 \times 10^{-3}$ M, respectively. The products of 7-ACA deacetylation were weak competitive inhibitors, and a $K_i$ value of $5.0 \times 10^{-3}$ M was determined for acetate and of $3.6 \times 10^{-2}$ M for deacetyl-7-ACA. Weak product inhibition did not prevent the deacetylation reaction from going to completion. A 5-mg/ml solution of partially purified esterase completely hydrolyzed (>99.5%) a 24-mg/ml solution of 7-ACA in 3 h. Because of the kinetic properties and excellent stability, this enzyme may be useful in an immobilized form to prepare large quantities of deacetylated cephalosporin derivatives.

Esterases that hydrolyze cephalosporins to deacetylcephalosporins and acetate (Fig. 1) have been detected in a variety of biological tissues.

Cephalosporin esterase activity has been found in citrus peels (10) and wheat germ (Belgium patent 671,692, Glaxo Laboratories Ltd., 1966), as well as in various bacteria and actinomycetes (3). It is also present in mammalian tissues, where it appears to be most prevalent in the liver and kidney (20). Cephalosporins with substituents at the C7 position, e.g., 7-(5-amino-5-carboxyvaleramido)cephalosporanic acid (cephalosporin C) and 7-(thiophene-2-acetamido)cephalosporanic acid (cephalothin), are also substrates of the enzyme.

The activity and toxicity of the cephalosporin molecule can be altered by changing substituents at the C7 and C3′ positions. Many modifications can be made at the 3′ position by nucleophilic displacement of the acetyl group. However, to make other modifications at this position, it is necessary to first remove the acetyl group. Chemical hydrolysis of acetyl from cephalosporins occurs in low yield because at alkaline pH values the Δ2 double bond migrates to the Δ1 position (19), and at acidic pH values lactone formation occurs between the 3′-hydroxymethyl and the adjacent carboxyl group (13). Thus, enzymatic hydrolysis, at pH 7.0, is the most effective method of preparing deacetylated derivatives in high yield.

Although several processes have been described for enzymatic cephalosporin deacetylation (24; Belgium patent 671,692; U.S. patent 3,304,236, J. Nüesch, Riehen, and H. Bickel, 1967), very little is known about these enzymes. Our studies were initiated to assess the potential utility of an esterase for preparation of deacetylated cephalosporins. As an initial step, the physical properties and kinetic behavior of a cephalosporin acetylesterase produced by Bacillus subtilis were determined.

MATERIALS AND METHODS

Microorganism. The microorganism used in these studies was B. subtilis WRRL-B-558 (from the Western Regional Research Laboratory, Albany, Calif.). Stock cultures were maintained on Trypticase soy agar (BBL, Cockeysville, Md.) slants. The microorganism was cultivated in 250-ml Erlenmeyer flasks containing 50 ml of Trypticase soy broth. The flasks were inoculated with a loop transfer from a slant and incubated at 30°C on a rotary shaker (250 rpm, 2.5-inch [ca. 6.4-cm] stroke). Growth was monitored by optical density measurements. A 2.0-ml sample of the culture was diluted with 38 ml of
water, and the optical density was measured at 660 nm (1-cm light path) in a Beckman model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Recovery and partial purification of the enzyme. The cephalosporin acetylase was precipitated from 1 liter of supernatant from 12- or 22-h cultures by adding solid ammonium sulfate to 85% saturation. The precipitate was recovered by centrifugation and was dialyzed at 4 C against 16 liters of 0.005 M potassium phosphate buffer (pH 7.0) by using a 100-ml 6/100-1 hollow-fiber beaker with a nominal molecular weight cut-off of 5,000 (Bio-Rad Laboratories, Richmond, Calif.). The dialyzed enzyme was lyophilized and then stored at 4 C where it remained stable for over 1 year. For isolation of the enzyme from the biomass, cells from 400 ml of medium were collected by centrifugation, washed, and suspended in 20 ml of 0.005 M potassium phosphate buffer, pH 7.0. The cells were treated with lysozyme (8) and disrupted with a French pressure cell (American Instrument Company, Silver Spring, Md.). After disruption, the enzyme was precipitated and recovered as described above.

A two- to threefold purification of the extracellular esterase was achieved by dissolving the lyophilized enzyme in deionized water and collecting the protein that precipitated between 60 and 80% of the ammonium sulfate saturation. An additional fivefold purification was obtained by removing low-molecular-weight peptides and proteins by ultrafiltration. A 2-mg/ml solution of esterase was placed in a 200-ml model 202 Amicon ultrafiltration cell containing a Diaflo PM 10 membrane with a nominal molecular weight cut-off of 10,000 (Amicon Corporation, Lexington, Mass.). The cell was pressurized with nitrogen (12 lb/in^2), and 1.5 liters of phosphate buffer was passed through. The esterase remained in the cell and was recovered by lyophilization. The ammonium sulfate fractionation and ultrafiltration steps were conducted in dilute phosphate buffer (0.005 M) to facilitate subsequent esterase assay by pH stat titration.

Enzyme molecular weight was estimated by gel filtration chromatography with a Sephadex G-200 gel. A 0.5-ml sample containing 20 mg of protein was applied to a column (2.5 by 50 cm; gel bed = 189 ml). The gel was equilibrated with 0.025 M potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl and 0.02% NaN3. The sample was eluted with the latter buffer system at a flow rate of 7.0 ml/h. The gel was calibrated with proteins of known molecular weight, and their elution volumes were established by protein assay of effluent fractions. The void volume, 46.25 ± 0.5 ml, was determined from the elution volume of dextran blue 2000. Esterase activity was determined by pH stat titration, with cephalothin (5.0 mg/ml) as the substrate.

Esterase and protein assay. The cephalosporin acetylase was assayed by continuous pH stat titration of the acetic acid liberated during the reaction. The system consisted of a Radiometer TTT11 automatic titrator, ABU 11T 0.25-ml buret, SBR3 recorder, and TTA31 reaction vessel (The London Company, Cleveland, Ohio). The reaction vessel had a 2.0-ml working volume and was maintained at 25 C with a water jacket. During the assay, a stream of humidified nitrogen gas was maintained over the surface of the reaction mixture. In the absence of the nitrogen stream, the solutions adsorbed CO2 from the atmosphere, which lowered the pH and triggered the addition of titrant. The titrant was a standardized solution of 0.004 N KOH, and the sensitivity of the system allowed the detection of the liberation of less than 1 nmol of protons/min per ml.

Cephalosporins in solution undergo a slow non-enzymatic hydrolysis that releases protons (12, 25). This hydrolysis, which is pH and temperature dependent, appears to be a first-order reaction at 25 C and pH 7, with a rate constant of 0.005/h. The contribution of non-enzymatic hydrolysis to the total measured reaction rate was determined by measuring the rate of titrant addition, under assay conditions, in the absence of enzyme. A 0.1-ml solution of esterase, usually containing 0.5 mg of protein, was then added to initiate the reaction. The non-enzymatic rate was subtracted from the total rate to obtain the net rate due to esterase-catalyzed hydrolysis.

Protein was determined spectrophotometrically by measuring absorption at 260 and 280 nm in a Beckman DB spectrophotometer. The conversion factor of Warburg and Christian (25) was used to convert absorbance to protein concentration.

Units and specific activity. A unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 10 nmol of 7-thiophene-2-acetamido-cephalosporanic acid (cephalothin) per 1 min at 25 C (pH 7) at a cephalothin concentration of 5.0 mg/ml. Specific activity was expressed as units per milligram of protein.

Bioautography. Deacetyl-7-aminocephalosporanic acid (deacetyl-7-ACA) and 7-ACA were resolved by descending paper chromatography, using Whatman no. 1 paper buffered with 0.075 M potassium phosphate buffer, pH 4.0. Papers were developed for 16 h in methanol-propanol-water (6:2:1, vol/vol/vol). After drying, the papers were sprayed with a 2% solution of NaHCO3 and then dipped into a solution of petroleum ether containing 0.3% phenoxyacetyl chloride. The acylated cephalosporins on the paper were detected by overlaying the chromatograms on agar media seeded with B. subtilis ATCC 6633. In this system, the Rf values for deacetyl-7-ACA and 7-ACA were 0.28 and 0.46, respectively. By spot 5
μg of a deacetyl-7-ACA/7-ACA mixture on the chromatograms, it was possible to detect less than 0.5% 7-ACA.

**Substrates.** Cephalothin and 7-ACA were obtained from the Lilly Research Laboratories. Cephalothin was 92% pure and 7-ACA, which was supplied as the hydrochloride, was 87% pure.

**RESULTS**

Enzyme production and recovery. The cephalosporin acetyesterase activity in the biomass and culture supernatant of *B. subtilis* was assayed at two points during cultivation: (i) near the end of the exponential growth phase, and (ii) 10 h after exponential growth ceased (Fig. 2). More activity was present after 22 h of cultivation than near the end of the exponential growth phase, and at both times most of the enzyme activity was associated with the biomass fraction. The enzyme was routinely recovered after 22 h of cultivation from the culture supernatant. Recovery from the supernatant obviated the need for cell disruption and nucleic acid removal. The specific activity of the initial ammonium sulfate precipitate was 0.8 to 1.0 U/mg of protein, and this was increased to 10.0 to 11.0 U/mg of protein in 70% yield by ammonium sulfate fractionation and ultrafiltration.

**pH and temperature optima.** Initial studies to determine the temperature and pH optima revealed that non-enzymatic substrate hydrolysis contributed significantly, particularly at alkaline pH values and elevated temperatures, to the total rate measurements. The contribution of non-enzymatic hydrolysis is shown in Fig. 3 and 4. When net rates were calculated, the pH optimum was 7.0 (Fig. 3) and the temperature optimum was between 40 and 50 C (Fig. 5).

Enzyme assays and kinetic measurements, nevertheless, were made at 25 C to: (i) minimize temperature inactivation of the enzyme, and (ii) increase the accuracy of the data by keeping the rate of non-enzymatic hydrolysis a small part of the total rate.

**Reaction kinetics.** Initial experiments established that measured reaction rates (corrected for non-enzymatic hydrolysis) were linearly dependent on enzyme concentration over the range tested (0.1 to 5.0 mg/ml). The dependence of the reaction rate on cephalothin concentration was determined. A double-reciprocal plot of the data produced a straight line that extrapolated to a *Kₘ* value of 8.3 × 10⁻² M (Fig. 6). A similar relationship was found with the substrate 7-ACA (Fig. 7), but the *Kₘ* value was less, 2.8 × 10⁻² M.

In a process for deacetylating cephalosporins, it is desirable that the reaction go to completion (i.e., all of the substrate is hydrolyzed). An important determinant is the effect of the reaction products on the reaction rate. Initial rate measurements were made at different 7-ACA concentrations in the presence and absence of added amounts of either deacetyl-7-ACA or acetate. Double-reciprocal plots of the data indicated that both reaction products are weak inhibitors; the *Kᵢ* of acetate was 5 × 10⁻² M and the *Kᵢ* of deacetyl-7-ACA was 3.6 × 10⁻² M. The data extrapolated to the same *Vₘₐₓ* values, suggesting that the inhibition is competitive.

**Enzyme molecular weight and stability.**

The molecular weight of the esterase was estimated by gel filtration with Sephadex G-200. The elution of the esterase from the Sephadex column is shown in Fig. 8. The elution volumes of proteins of known molecular weight were measured in triplicate. From the elution volumes, the distribution coefficients (*Kₐᵥ*) were calculated and plotted as a function of the log molecular weight (Fig. 9). The resultant standard curve indicates that cephalosporin acetyesterase has a molecular weight of 190,000.

The stability of the enzyme was assessed by measuring esterase activity as a function of time during heating at various temperatures. The results (Fig. 10) show that the enzyme is very stable and exhibits the unique property of being protected from inactivation by 1.0 M phosphate buffer, pH 7. The stability of the unbuff-
Fig. 3. Effect of pH on cephalothin deacetylation by B. subtilis cephalosporin acetylase. The reactions were measured at 25°C, with 0.25 mg of esterase per ml and 5.0 mg of cephalothin per ml. Symbols: ○, rate of non-enzymatic deacetylation of cephalothin; ×, total reaction rate (non-enzymatic plus enzymatic deacetylation); ○, net rate of cephalothin deacetylation (total rate minus non-enzymatic rate).

Fig. 4. Effect of temperature on non-enzymatic cephalothin deacetylation (○) and on the total rate of cephalothin hydrolysis (●) (enzymatic plus non-enzymatic hydrolysis). The reaction mixtures contained 1.0 mg of esterase per ml and 5.0 mg of cephalothin per ml.

Fig. 5. Net rate of cephalothin deacetylation by B. subtilis cephalosporin acetylase. Net rate was obtained by subtracting the rate of non-enzymatic hydrolysis from the total reaction rate (enzymatic plus non-enzymatic). Data were obtained from Fig. 4.

Fig. 6. Double-reciprocal plot of reaction velocity and substrate concentration for cephalothin deacetylation by B. subtilis cephalosporin acetylase. From the plot $V_{max} = 3.3 \times 10^{-8}$ mol/min per ml and $K_m = 8.3 \times 10^{-3}$ M.
lent of 7-ACA present initially. At the end of each reaction, deacetyl-7-ACA and unreacted 7-ACA were recovered by isoelectric precipitation at pH 4.2. Nuclear magnetic resonance (100 MHz) spectroscopy confirmed the structure of the product, and no peak was detected at 2.1 δ characteristic of the acetyl group of 7-ACA. The absence (<0.5%) of 7-ACA also was confirmed by descending paper chromatography and bioautography.

**DISCUSSION**

Members of the genus *Bacillus* produce many enzymes of commercial importance. These in-clude proteases, amylases, and pectinases (4), as well as beta-lactamases and penicillin and cephalosporin aminohydrolases (5). Enzymes produced by *Bacillus* species have also been studied because of their possible roles in the sporulation process. Three distinct hydrolytic enzymes have been recovered from sporulating cultures of *B. subtilis*. One is an alkaline protease that exhibits esterase and protease activity (6, 15, 22), another is a metallo-protease that

![Fig. 7. Double-reciprocal plot of reaction velocity and substrate concentration for 7-ACA deacetylation by *B. subtilis* cephalosporin acetyl esterase. From the plot \( V_{\text{max}} = 4.15 \times 10^{-8} \text{ mol/min per mg} \) and \( K_m = 2.8 \times 10^{-3} \text{ M} \).](image)

![Fig. 8. Elution profile of *B. subtilis* cephalosporin acetyl esterase from a Sephadex G-200 column. Symbols:  O, units of esterase activity;  o, adsorption at 280 nm.](image)

![Fig. 9. Molecular weight estimation of *B. subtilis* cephalosporin acetyl esterase by chromatography on Sephadex G-200.](image)

![Fig. 10. Effect of temperature on the inactivation of *B. subtilis* cephalosporin acetyl esterase. The esterase solution (1 mg/ml) was heated for the times and temperatures indicated, quickly cooled to 25 C, and assayed with 5 mg of cephalothin per ml.](image)
does not hydrolyze ester substrates (11, 14), and the third is an acidic protease with high esterase activity (2, 7, 16, 21). More recently, Higerd and Spizizen (8) isolated two esterases, designated esterase A and esterase B, from a \textit{B. subtilis} culture. They speculated that these enzymes may be identical to the acid protease described above and to an esterase isolated from \textit{B. megaterium} by Millet et al. (17, 18). The cephalosporin acetyltransferase described in the present study has several characteristics similar to esterase A isolated by Higerd and Spizizen. Both enzymes are stable when heated at 60°C and unstable when heated at 70°C. Also, both esterases are stabilized at 80°C by 1.0 M phosphate buffer. Neither enzyme hydrolyzed casein and both hydrolyzed \(\beta\)-naphthol acetate (Abbott and Fukuda, unpublished data). Although there are several similarities, the two enzymes may not be identical. Higerd and Spizizen (8) estimated the molecular weight of esterase A to be 160,000 by chromatography on Sephadex G-150, but our studies indicated that the cephalosporin acetyltransferase had a molecular weight of 190,000 (Sephadex G-200).

Jeffery et al. (10) reported that a cephalosporin acetylase obtained from citrus peels hydrolyzed cephalosporin C and triacetin, with \(K_m\) values of \(4.7 \times 10^{-3}\) and \(32 \times 10^{-3}\) M, respectively. The \(K_m\) values obtained with the \textit{B. subtilis} esterase and cephalosporin substrates were similar to those measured by Jeffery et al. However, their \(K_m\) value for triacetin is about 2 orders of magnitude greater than the value of \(0.46 \times 10^{-3}\) M (unpublished data, Abbott and Fukuda) measured with the bacterial enzyme and triacetin. This result indicates that the citrus and bacterial esterases are quite different.

The kinetic studies of the cephalosporin acetylase revealed several features of the enzyme that make it attractive for producing deacetylated cephalosporin. The pH optimum is near 7.0, where substrate losses due to non-enzymatic hydrolysis are minimum. Although the temperature optimum occurs in a range where non-enzymatic hydrolysis is substantial, the esterase exhibits about 68% of the maximum activity at 25°C, where non-enzymatic hydrolysis is much less. Another positive factor is the absence of substantial product inhibition on the reaction rate. The weak inhibition observed was not sufficient to prevent complete (>98%) hydrolysis of the substrate, although it undoubtedly extended the reaction time course.

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