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Arylamidase of *Cephalosporium acremonium* and Its Specificity for Cephalosporin C

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Three aggregational forms of arylamidase are produced by *Cephalosporium acremonium*. The exocellular enzyme, with an approximate molecular weight of 60,000, was purified 300-fold by diethylaminoethyl cellulose chromatography, gel filtration, and gel electrophoresis. With L-leucyl-β-naphthylamide as the substrate, the K_m is 4.2×10^{-4} M; the optimum *p*H, 7.7; and the temperature optimum, 35 C. The enzymatic hydrolysis of L-leucyl-β-naphthylamide is inhibited by a number of cephalosporins, whereas a variety of penicillins show no effect. Alternatively, the enzyme specifically catalyzes the β-lactam hydrolysis of a number of cephalosporins; a number of penicillins are resistant. The K_m for cephalosporin C is 9.09×10^{-4} M.

Arylamidases have been reported in bacteria (1, 21), fungi (20), and mammalian tissue (2, 11, 16) and identify those enzymes that hydrolyze amino acid arylamides, such as L-leucyl-β-naphthylamide (LN; reference 19). A biological function is usually not assigned to them unless they can be shown to hydrolyze a substrate of physiological relevance.

The fungus *Cephalosporium acremonium*, which produces two peptide antibiotics, penicillin N and cephalosporin C, is known to elaborate two peptidases, a type I acylase and a proteinase. The acylase hydrolyzes phenoxymethylpenicillin to 6-aminopenicillanic acid (6APA) and phenoxyacetate (6, 7), whereas the proteinase attacks casein and various synthetic peptides, for example, benzoyl-L-tyrosine (18; F. M. Huber et al., Mycopathol. Mycol. Appl.).

Although neither enzyme catalyzes the degradation of the penicillin N or cephalosporin C, it was observed in these laboratories that spent media from certain strains of *C. acremonium* were destructive to the antimicrobial activity of cephalosporin C. The same spent media had significant levels of arylamidase activity which coincided with the instability of the antibiotic. An opportunity was thus presented to evaluate *C. acremonium* arylamidase in relation to its specificity toward penicillin N and cephalosporin C.

This report deals with culture conditions of *C. acremonium* requisite to arylamidase production and with the partial separation and purification, kinetic properties, and specificity of the enzyme.

MATERIALS AND METHODS

Organism and culture conditions. Wild-type *C. acremonium* Corda, ATCC 11,550, and a mutant M8650 were obtained from the National Research and Development Corp., London, England, and a mutant M8650-3 was the same as described in an earlier report (9). The cultures were maintained on agar slants of a modified LePage and Campbell medium (4). The basal medium used for all experiments contained, in grams per liter of deionized water: sucrose, 36.0; glucose, 27.0; KH_2PO_4, 0.15; K_2HPO_4, 0.21; $MgSO_4 \cdot H_2O$, 0.09; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.02; $CaCl_2$, 0.03; $MnSO_4 \cdot H_2O$, 0.004; $ZnSO_4 \cdot 7H_2O$, 0.03; $CuSO_4 \cdot 5H_2O$, 0.002; and $CaCO_3$, 10.0. This medium (40 ml) was dispensed into Erlenmeyer flasks (250 ml), and methyl oleate (0.5 ml) was added; the flasks were stoppered with cotton plugs and autoclaved (121 C, 15 psi, 15 min). Nitrogen supplements were filter-sterilized and added aseptically to the basal medium. Shaken flasks always contained a total of 50 ml in the aqueous phase including the inoculum.

Seed flasks containing the basal medium and L-asparagine (10.0 mg/ml) as the nitrogen source were inoculated with the spores and incubated (25 ± 1 C) on a rotary shaker (250 oscillations/min), harvested after 5 days, and washed twice with sterile water. Suspensions of washed mycelium were adjusted so that a stock suspension contained a dry weight of 0.05 ± 0.01 g/ml.

For physiological studies, the stock cell suspension (5.0 ml) was inoculated into fresh basal medium supplemented with various nitrogen sources as required. The shaken flasks were incubated in the same manner as described above.

Cephalosporin C and penicillin N assay. Culture medium was filtered and assayed for cephalosporin C and penicillin N content by the microbiological plate

assay method with *Salmonella gallinarum* ATCC 9184 as the test organism (14). The assay was insensitive to cephalosporin P, another antibiotic present in the culture medium. In most instances, total activity contributed by cephalosporin C and penicillin N was measured; however, differential activity was assayed as required by selectively destroying penicillin N with penicillinase (Riker Laboratories, Northridge, Calif.).

Arylamidase assay. Arylamidase activity was measured by the liberation of β-naphthylamine from the synthetic substrate LN (Sigma Chemical Co.). The reaction mixture contained 1.0 ml of the enzyme preparation, 0.5 ml of tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer (0.1 M, *p*H 8.0), and 0.5 ml of the substrate (1.37×10^{-3} M). After incubation at 35 C, the reaction was stopped by the addition of 1.0 ml of trichloroacetic acid (40%). The incubation mixture was centrifuged, and 1.0 ml of the supernatant fluid was assayed by coupling the liberated β-naphthylamine with *N*-(1-naphthyl)ethylenediamine by a previously described method (12). The resulting diazo compound was measured in a spectrophotometer (Beckman model DB) at 560 nm and quantitated with standard β-naphthylamine.

Enzyme activity was defined as micromoles of β-naphthylamine liberated per hour per milliliter of enzyme preparation. Specific activity was expressed in terms of protein (milligram) equal to a bovine serum albumin standard as measured by the procedure of Lowry et al. (15).

TABLE 1. *Effect of nitrogen sources in the culture medium on arylamidase activity and antibiotic potency by C. acremonium*

Nitrogen source: methionine[a]	Total antibiotic potency: penicillin N and cephalosporin C (μg/ ml)	Arylamidase activity (units/ml)	
		Cells	Culture medium
DL-Alanine	<600	40.5	95.9
L-Arginine	780	54.7	32.8
DL-Aspartic acid	<600	53.4	73.3
L-Asparagine	**1,215**	6.7	32.3
L-Cystine	<600	30.7	29.3
L-Glutamic acid	450	27.9	35.7
L-Glutamic acid ethyl ester	555	51.2	19.0
L-*N*-acetylglutamic acid	<600	24.4	**293.0**
L-Glutamine	**1,945**	52.3	55.0
Glycine	<600	36.7	**117.5**
L-Leucine	<600	33.8	**108.0**
L-Lysine	<600	43.3	80.4
L-Phenylalanine	885	28.7	40.0
L-Proline	555	51.5	49.1
Nitrate (sodium)	510	29.1	**103.9**
Taurine	510	14.7	**107.2**
L-Tryptophan	**1,545**	47.2	55.2
Water	<600	26.1	**473.6**

[a] Methionine concentration, 0.01 M; other additives, 0.01 M in nitrogen.

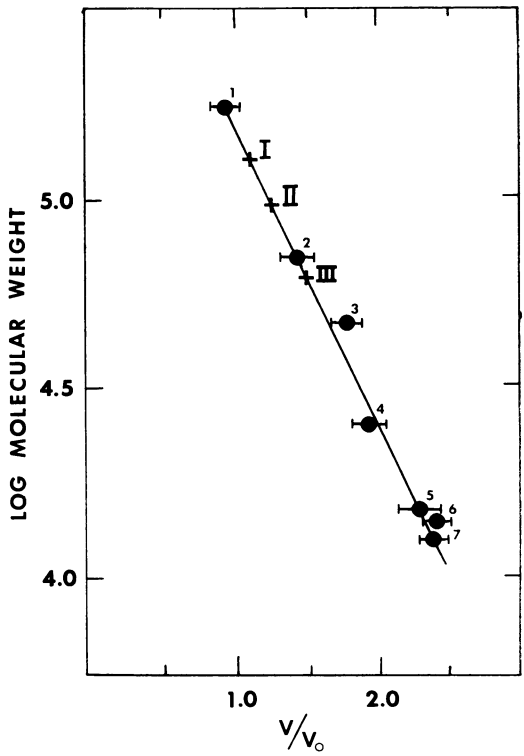

FIG. 1. *Gel filtration of C. acremonium arylamidase. A Sephadex G-100 column (2 by 68 cm) was used. Samples (1.0 ml) were added to the column and eluted with Tris-hydrochloride buffer (0.045 M, pH 7.0; KCl, 0.10 M). V is the elution volume of the sample and V_o is the void volume of the column. Standards and their molecular weights: dextran blue, 10^6 (1); bovine serum albumin, 67,000 (2); ovalbumin, 45,000 (3); chymotrypsinogen, 25,000 (4); α-lactoglobulin, 15,000 (5); ribonuclease, 13,700 (6); cytochrome c, 12,400 (7).*

Cephalosporinase activity. The activity of arylamidase toward cephalosporin substrates was assayed by the microbiological plate assay method described as above. Kinetic data were obtained by periodically removing samples from the reaction mixture and plating them on the seeded petri plates. Rates of cephalosporin destruction by arylamidase were compared with incubation mixtures of substrate in the absence of enzyme. β-Lactam content of incubated cephalosporin C was measured by the hydroxamate method (3).

Chromatography and bioautography of reaction mixtures. The liberation of leucine from LN and the destruction of cephalosporins by arylamidase were examined by paper chromatography. Descending chromatography was performed on Whatman no. 1 paper with 85% aqueous acetone as the solvent. After separation, the location of cephalosporin C was visualized either by its ultraviolet absorbance under a dark lamp or by ninhydrin reactivity. Antimicrobial activity was checked by placing the chromatography

sheet on a seeded agar plate for 5 min, removing the sheet, and incubating the plate overnight at 37 C. Cephalosporin C and desacetylcephalosporin C were located by appropriate zones of inhibition. To test for acylase activity, the chromatography sheet was first dipped in 0.1% $NaHCO_3$ solution, air-dried, and then sprayed with phenoxyacetylchloride (0.1% in acetone) and air dried again before placing on the seeded agar plate. Standards of 6-APA and 7-aminocephalosporanic acid (7ACA) were acylated on the paper after chromatography.

Partial purification of arylamidases. Washed mycelium was mixed in Tris-hydrochloride buffer (0.1 M, *p*H 7.0) and centrifuged at 2,000 × *g*; an equal volume of acid-washed glass beads (type 100-5005, Superbrite, 3 M Co., St. Paul, Minn.) was added. The mixture was transferred to an ice-cold Omni-Mixer homogenizer (Ivan Sorvall, Inc., Norwalk, Conn.), the air was replaced with nitrogen, and the cells were disrupted for 10 min at a rheostat setting of 70. The extract was centrifuged (model RC-2, with SS-34 head, Ivan Sorvall, Inc.) at 2,000 × *g* for 10 min, the centrifuged solids were discarded, and the supernatant fluid was dialyzed overnight against Tris-hydrochloride buffer (0.01 M, *p*H 7.0). The volume of the dialyzed supernatant fluid was reduced by placing the dialysis tube on solid Carbo-Wax (polyethylene glycol compound, 20 M, Union Carbide Corp., New York). Arylamidase was purified further by repeated application, elution, and volume reduction from a diethylaminoethyl (DEAE) cellulose column prepared as described previously (10). Homogeneity and molecular weight of the partially purified enzymes were determined from their elution profile from a gel filtration column (Sephadex, Sigma Chemical Co., St. Louis, Mo.) or by electrophoresis through polyacrylamide gel (8). Purification of the exocellular arylamidase was accomplished by adsorption of the culture medium filtrate on DEAE cellulose (equilibrated with Tris-hydrochloride buffer, 0.01 M, *p*H 7.0), washing with 0.4 M buffered KCl, and then eluting the enzyme with a higher salt concentration (Tris-hydrochloride buffer, 0.01 M, *p*H 7.0; KCl, 0.5 M).

Isotope studies. Cephaloglycin labeled with ^{14}C in the acylcarbonyl group was synthesized by Hugh R. Sullivan of these laboratories and had a specific activity of 60 μCi/mg. The location of labeled components in Sephadex column eluates was determined with a Packard Tri-Carb liquid scintillation spectrophotometer. The counts were measured in vials of the aqueous sample (0.1 ml) and scintillation fluid (20.0 ml). The scintillation fluid was composed of 4 g of 2,5-diphenyloxazole (PPO) and 4 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter of a 7:3 (v/v) toluene-ethoxyethanol solvent. Chromatograms containing radioactive components were counted on a Vanguard automatic scanner, model 880.

RESULTS

Initial data on arylamidase production by C. acremonium. After the detection of arylamidase activity in complex culture medium and in washed cells of *C. acremonium*, a series of amino acids was tested as the nitrogen sources. Five-day-old cells were washed three times with water and then with buffer to give a final packed cell volume of 0.5 ml. This volume of cells and 0.5 ml of the original culture fluid were assayed for arylamidase activity. The influence of the various nitrogen sources on the production of arylamidase activity and relative distribution between whole cells and culture medium are shown in Table 1.

These data indicated that the relative distribution of the enzyme activity between cells and broth varied when the cells were grown on different nitrogen sources. Methionine as the sole nitrogen source was the most effective amino acid in stimulating arylamidase activity. For significant antibiotic potencies to be produced, however, certain other amino acids were required as supplements. It was observed at this point that, in general, an inverse relationship between antibiotic potency and arylamidase activity might exist. Amino acids, such as asparagine,

TABLE 2. *Purification of arylamidase from C. acremonium culture medium*[a]

Step	Vol (ml)	Arylamidase activity (units/ml)	Protein concn (mg/ml)	Specific activity (units/ml of protein)	Step recovery (%)[b]	Step purification (fold)
1	1,905	554	11.5	48.2		
2	2,000	1,186	2.3	515.6	224	10.7
3	215	1,280	1.4	915.0	11.6	1.8
4	105	2,586	2.8	923.6	98.6	−1.0
5	20	2,862	0.2	14,310.0	21.1	15.5

[a] Description of steps: (1) dialyzed culture medium, 0.01 M Tris-hydrochloride buffer, *p*H 7.0; (2) eluate (0.01 M Tris-hydrochloride, 0.50 M KCl) from DEAE-cellulose column after adsorbing dialyzed culture medium and discarding a previous column wash (0.01 M Tris-hydrochloride, 0.40 M KCl); (3) repeat of step (2); (4) concentration of eluate in dialysis tubing versus solid Carbo-Wax; (5) chromatography of step (4) material on Sephadex G-100 column, combining peak fractions.

[b] Total per cent step recovery from step 1, 5.4%; total step purification, 296.8-fold.

glutamine, and tryptophan, evoked β-lactamase potencies greater than 1,200 μg/ml, whereas the arylamidase activity in the medium was less than 60 units/ml. Those combinations of amino acids that stimulated arylamidase activity evoked low potencies of the antibiotics.

Since it was of interest to determine whether the endocellular and exocellular activites were representing the same enzyme molecule, preliminary kinetic studies showed that the arylamidase activity of the cell-free supernatant fluids and culture medium filtrates had the same *p*H and temperature optimum and the same K_m. However, gel filtration of the preparations through Sephadex G-100 revealed different species of the enzyme. As depicted in Fig. 1, three aggregational forms were visualized, with fractions I and II being derived from cell-free supernatant fluids and fraction III (fr III) from the exocellular culture medium. The same size distribution was noted whether cell-free supernatant solutions and exocellular culture medium were premixed or run separately over the column. The approximate molecular weights of 120,000, 90,000, and 60,000 for the three fractions suggest, but do not prove, the existence of one enzyme with a minimum molecular weight of about 30,000. (Work is in progress to evaluate the intracellular arylamidases.)

Further studies reported herein deal with the exocellular arylamidase, fr III, because of easy access to the culture medium, its smaller molecular weight, and because it is present in the culture medium during the period of penicillin N and cephalosporin C excretion by *C. acremonium*.

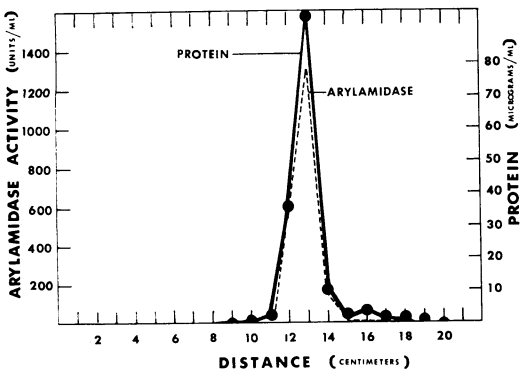

FIG. 2. *Electrophoresis of purified C. acremonium arylamidase, fraction III. Electrophoresis was conducted on small-pore gel (9), polyacrylamide, 0.8 by 10.5 cm; 300 volts, 2.8 ma, 4.5 hr. The system was buffered with Tris-glycine (Tris, 0.05 M; glycine, 0.04 M; pH 8.0). Enzyme units (2,049) were applied to gel; recovery of 95% was made by slicing gel and eluting with water.*

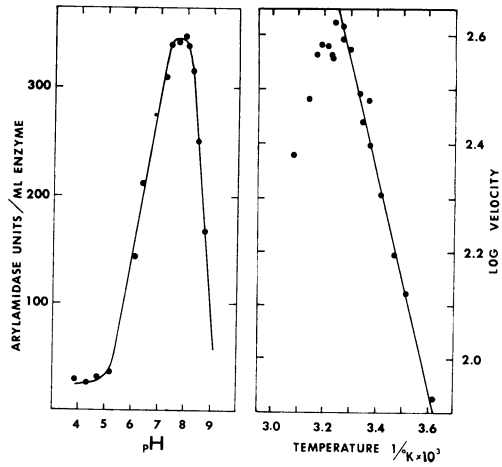

FIG. 3. *pH and temperature optimum of C. acremonium arylamidase fraction III. pH maximum was determined in a manual pH stat. Temperature optimum was 35 C, with Arrhenius energy of activation (E_a) of 9.20 kcal.*

TABLE 3. *Effect of penicillins and cephalosporins on arylamidase, fraction III, with L-leucyl-β-naphthylamide (LN) as substrate*

Additive (10^{-4} M)	Inhibition (%)
Cephalosporin C	81.0
Penicillin N	0
Cephaloglycin	75.0
Cephalexin	74.6
Ampicillin	0
7-Phenoxymethylcephalosporin	70.5
7-Phenoxymethyldesacetoxycephalosporin	61.1
Phenoxymethylpenicillin	0
Keflin	41.0
Loridine	22.2
Thienylmethylpenicillin	0
Benzylcephalosporin	37.6
Benzylpenicillin	0
Chloromethylpenicillin	0
β-Hydroxyphenoxymethylpenicillin	0
Methicillin	0
β-Methylphenoxymethylpenicillin	0
Oxacillin	0
Phenethicillin	0
Phenoxypropylpenicillin	0
Phenylmercaptomethylpenicillin	0

Stability studies with arylamidase fr III. Dialysis of the culture medium against Tris-hydrochloride buffer (0.01 M, *p*H 7.0; KCl, 0.5 M) caused a 58% increase in specific activity. The enzyme activity in filter-sterilized spent culture medium was stable for at least 3 months either frozen or at 5 C. At least 95% of the arylamidase

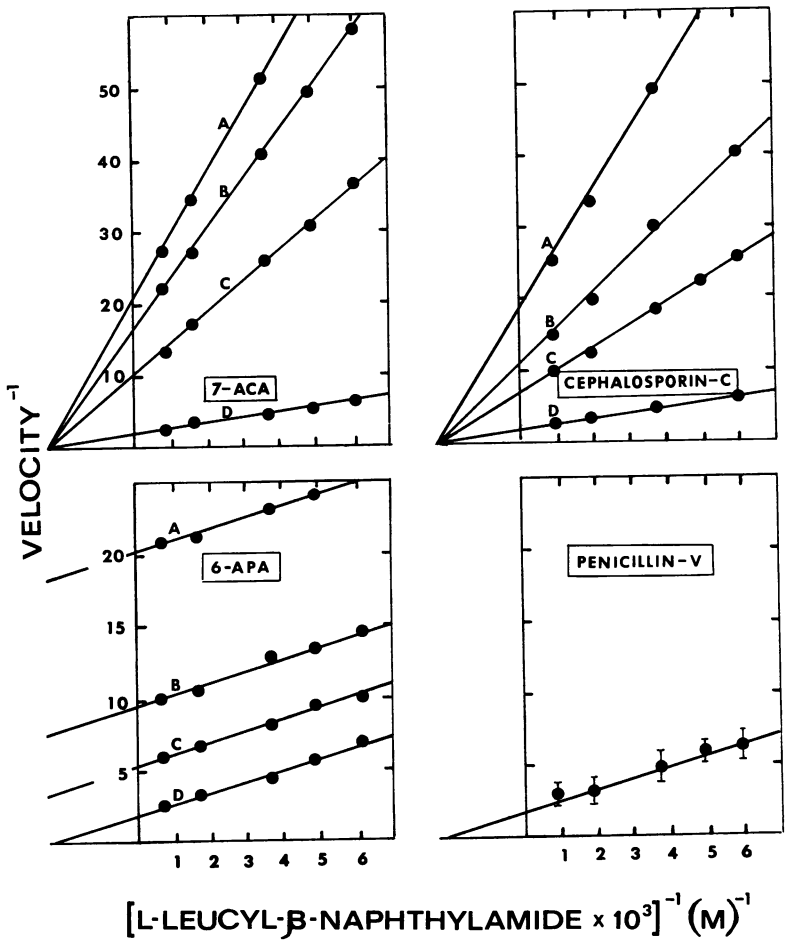

FIG. 4. *Inhibition of C. acremonium arylamidase. Kinetic experiments were performed at pH 8.0, 35 C, with LN as substrate. Concentrations of inhibitors in μmoles/ml: 7ACA, 6.25, (A); 3.13, (B); 1.25, (C); 0, (D); K_i, 4.0 × 10^{-4} M; cephalosporin C, 0.57, (A); 0.23, (B); 0.11, (C); 0, (D); K_i, 2.4 × 10^{-5} M; 6APA, 2.31, (A); 1.15, (B); 0.46, (C); 0, (D); K_i, 6.7 × 10^{-4} M; penicillin V, 1.28, 0.64, 0.26, and 0. The K_m for LN, 4.2 × 10^{-4} M.*

activity remained after 2 weeks at 25 ± 3 C. The half-life of arylamidase fr III in filter-sterilized culture medium was 2.3 min at 89 ± 1 C.

Partial purification of arylamidase fr III. Culture medium filtrates from cells grown on basal plus glutamate medium were used as starting material for purification of the arylamidase. Table 2 shows the steps in purification of the enzyme. The partially purified preparation (300-fold) was filtered through Sephadex G-100 and subjected to polyacrylamide gel electrophoresis to test the degree of homogeneity. Essentially all measurable contamination was absent (Fig. 2). No further purification could be detected by gel electrophoresis. The specific activity of the arylamidase band when eluted from the gel assayed 14,295 units/mg of protein, an overall 300-fold purification.

Kinetic studies with arylamidase fr III. The 300-fold purified enzyme was used for subsequent kinetic studies. Data for the *p*H and temperature optima are shown in Fig. 3. The K_m value for LN was 4.2 × 10^{-4} M. Similar compounds, such as *N*-γ-L-glutamyl-β-naphthylamide, β-naphthylacetate, β-naphthylpropionate, and β-naphthylbutyrate, did not serve as substrates for the enzyme. Of principal interest was the relation of the arylamidase to penicillin N and cephalosporin C, the normal peptide antibiotics found in the culture medium of *C. acremonium*. Initial experiments revealed that the arylamidase was inhibited by cephalosporin C and by other chemi-

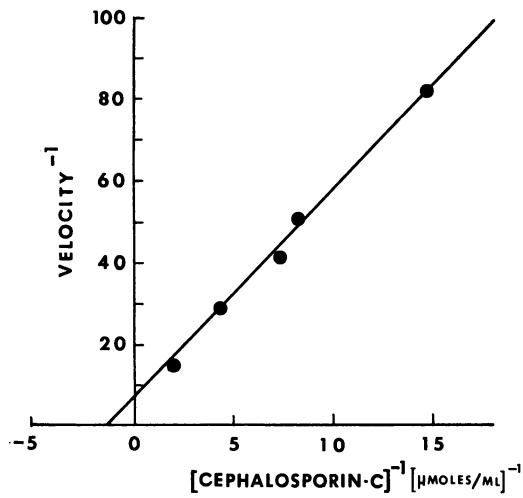

FIG. 5. *Degradation of cephalosporin C by arylamidase of C. acremonium. Cehpalosporin C and arylamidase were incubated at 35 C, pH 8.0. Rates were determined over a 3-hr period for each concentration of substrate and compared with appropriate controls.* $K_m = 9.09 \times 10^{-4}$ M.

cally derived cephalosporins and not affected by penicillin N and its analogues (Table 3). Penicillins, cephalosporins, and desacetoxycephalosporins, with common side chains, are grouped together where applicable in Table 3.

At this point, it was clear that the *C. acremonium* arylamidase was subject to a specific inhibition by cephalosporin derivatives which were common to each other with respect to the fused β-lactam-thiazoline ring structure. Differences in the 3-acetoxy function or 7-aminoacyl side chain did not appear to contribute to the inhibitory properties of the respective molecules.

A more detailed kinetic analysis was performed with cephalosporin C, 7ACA, phenoxymethylpenicillin, and 6APA. The data shown in Fig. 4 indicated that 7ACA and cephalosporin C evoked a noncompetitive inhibition with LN; 6APA was uncompetitive; and, as noted previously in Table 3, phenoxymethylpenicillin had no measurable effect. The inhibition of arylamidase by cephalosporin C explained why dialysis of culture medium filtrates containing cephalosporin C caused an activation of the enzyme. The inhibition of arylamidase by 6APA, but not by phenoxymethylpenicillin as well as other penicillins, suggested that the enzyme had no catalytic effect on the acyl side chain of these antibiotics. It was also observed that the antimicrobial activity of phenoxymethylpenicillin in the reaction mixture remained at a control level during the 1-hr incubation period. The reaction mixtures containing cephalosporin C and 7ACA, however, lost antimicrobial activity, suggesting that the arylamidase was not only inhibited by these molecules but was also catalyzing their destruction. No free α-aminoadipic acid or 7ACA was detected by chromatography in the reaction mixture, but it was observed that cephalosporin C had degraded to inactive components.

A direct assay of arylamidase action on cephalosporin C was performed by incubating the enzyme and the substrate under the conditions optimal for hydrolysis of LN. The data (Fig. 5) identify arylamidase, fr III, as a cephalosporin-specific β-lactamase. It should be noted that the decomposition route of cephalosporin C was not delineated to identify the exact intermediate modes of hydrolysis. The absence of hydrox-

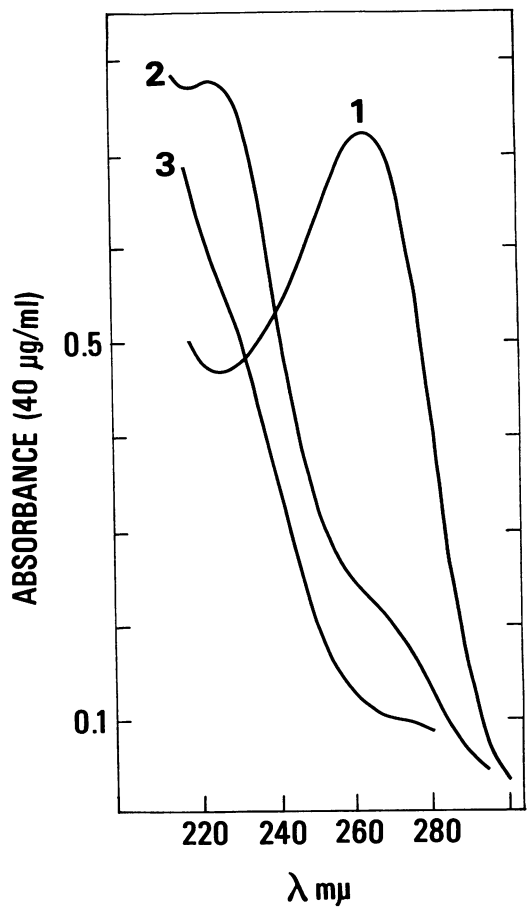

FIG. 6. *Changes in UV spectrum of cephalosporin C in presence of arylamidase and sodium hydroxide. Curves: cephalosporin C at pH 7.2, 25 C (1); cephalosporin C incubated for 24 hr at pH 10, adjusted to pH 7.2 (2); cephalosporin C after incubation with arylamidase III, 30 min, pH 7.2 (3).*

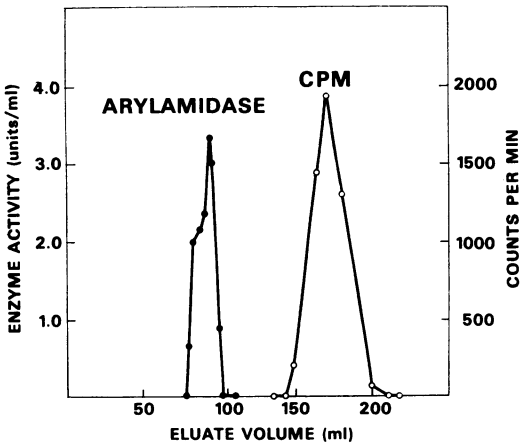

FIG. 7. *Gel-filtration of arylamidase-^{14}C-cephaloglycin mixture. Preincubated arylamidase (123 units)-^{14}C-cephaloglycin (1 mg; 60 μCi) mixture was applied and chromatographed through a Sephadex G-100 column.*

amate reactivity as well as the loss of 260-nm absorbance in the enzymatically hydrolyzed substrate, however, proved that the loss of β-lactam was one of the resultant effects of enzyme action. Figure 6 shows the shift in ultraviolet absorbance that takes place when cephalosporin C is incubated with arylamidase fr III.

Character of inhibition. The noncompetitive inhibition of arylamidase fr III by cephalosporins when LN was the substrate suggested two possible modes of interaction: (i) binding of the inhibitor to enzyme or (ii) catalytic chemical modification (i.e., inactivation) of the enzyme. To examine the nature of the interaction, arylamidase (123 units) was applied to the Sephadex G-100 column after incubating with the ^{14}C-cephaloglycin (1.0 mg; 60 μCi) for 1 hr at 37 C. Recovery from the column was 88% for a control enzyme preparation alone. After incubation with ^{14}C-cephaloglycin, the recovered enzyme was 45.9 units which represented a 50.6% inactivation by the substrate. As shown in Fig. 7, no binding between cephaloglycin and arylamidase was detected. This result indicated that intact antibiotic did not bind to the enzyme. The decrease in enzymatic activity after interaction with the substrate suggested that either the antibiotic and enzyme were inactivated through mutual interaction or that binding of a hydrolytic product which did not contain the label occurred.

To analyze the enzymatic degradation of cephaloglycin, the G-100 eluate containing enzymatically degraded cephaloglycin was first concentrated in vacuo and then chromatographed on paper. For comparison, standard solutions of cephaloglycin, desacetylcephaloglycin, and a sodium hyroxide-degraded sample of cephaloglycin were chromatographed separately and also mixed with the G-100 eluate. The radioactivity aligned with a component of the sample degraded with sodium hydroxide (Fig. 8). This component indicated the presence of sulfur and an amino group since it was ninhydrin- and iodine-reactive.

DISCUSSION

Arylamidase of *C. acremonium* has been shown to exist in three aggregational forms. The larger species are found associated with mycelium or are intracellular. A form of approximately 60,000 molecular weight is exclusive to the extracellular milieu. The role of the arylamidases in the metabolism of *C. acremonium* has not been demonstrated; however, the exocellular arylamidase has been shown to have hydrolytic specificity toward cephalosporin C.

When LN was used as a substrate, a number of cephalosporins were inhibitory to the reaction, whereas these same antibiotics when tested as enzyme substrates were inactivated by the arylamidase through β-lactam cleavage. The rapid initial changes detected by the ultraviolet absorption spectrum of cephalosporin C when incubated with *C. acremonium* arylamidase were essentially identical to that reported by Newton et al. (17) for β-lactamase II of *Bacillus cereus*. The penicillins tested were inert substrates. Thus, an exo-

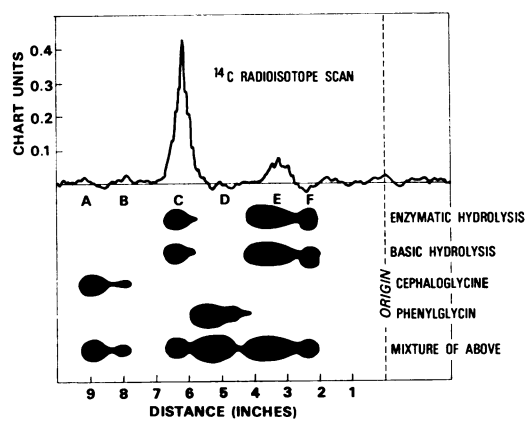

FIG. 8. *Paper chromatography and ^{14}C scan of cephaloglycin degradation. Paper chromatography was performed as in the text. Top part of figure shows ^{14}C scan of reaction products from arylamidase, fraction III action on ^{14}C-cephaloglycin. Letters indicate: (A) cephaloglycin, sulfur ⊕, ninhydrin ⊕; (B) desacetylcephaloglycin, sulfur ⊕, ninhydrin ⊕; (C) unknown sulfur ⊕, ninhydrin ⊕ spot; (D) phenylglycine, sulfur ⊖, ninhydrin ⊕; (E and F) unknown spots, sulfur ⊕, ninhydrin ⊕. Bioautography revealed only spots A and B to be antimicrobially active.*

cellular enzyme of *C. acremonium* M8650-3 is present in culture medium and catalytically destructive for one of the two antibiotics produced by the fungus.

The inertness of the penicillins and the inhibitory properties of 6APA describe a unique specificity of the enzyme. The exocellular arylamidase appears to be unable to attack the β-lactam of penicillins that are protected by 6-aminoacyl groups while readily attacking cephalosporins with like side chains. No detailed analysis of the 6APA-arylamidase interaction was performed to determine whether this molecule was hydrolyzed when acting as a substrate. Investigations by Chiang and Bennett (5), however, have shown that 6APA is a noncompetitive inhibitor of penicillin acylase of *B. megaterium*. Both 6APA and 7ACA also inhibit the penicillin acylase activity of *Actinoplanes utahensis* (Carver, *unpublished data*).

To examine the inhibitory properties of cephalosporins on arylamidase, ^{14}C-cephaloglycin was used as a substrate. Filtration of the partially inactivated enzyme through Sephadex G-100 failed to regenerate the initial enzyme activity and the dialysis was also ineffective. Although the partial inactivation of arylamidase by the substrate, as well as kinetic analyses, suggested substrate binding, chromatography of the reaction products proved that the intact substrate did not bind to the enzyme.

Inhibition of arylamidase by one of the products of the enzyme action appears to be most likely. The ^{14}C label of enzymatically hydrolyzed cephaloglycin aligned with a sodium hydroxide-degraded component of the same antibiotic which can be explained through the known mechanism described by Jeffery et al. (13).

Enzymatic hydrolysis of the β-lactam ring of ^{14}C-cephaloglycin would result in retention of the label in the resulting thiazole ring. The chromatography of the enzymatic reaction mixture showed that the ^{14}C label aligned with a sulfur- and amino group-containing compound not unlike the thiazole moiety produced by degradation of cephalosporin C. Unanswered is the possibility that a component of the product of the enzymatic reaction, other than a thiazole, was the actual inhibitor. The fact that the enzyme was not completely inactivated, even in the presence of substrate excess, suggests a finite number of binding sites whose allosteric effects are adequately remote from the catalytic site. The partially inhibited enzyme was capable of the continued hydrolysis of LN as well as cephalosporin C. If, in fact, no binding of an enzymatic product occurred, it is assumed that substrate-enzyme interaction so altered the catalytic or allosteric site so as to render the enzyme partially incompetent.

The specificity of the extracellular arylamidase toward one of the two sulfur-containing antibiotics in *C. acremonium* has important implications to the ultimate accumulation of cephalosporin C during its biosynthesis as well as its stability in subsequent filtered-culture medium. The influence of the enzyme on the accretion of cephalosporin C and penicillin N during the early period of antibiotic production in shaken flasks is not clear. However, after the cessation of antibiotic synthesis in shaken flasks, the ratio of cephalosporin C to penicillin N decreases as a function of time.

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