

# Purification and Properties of an Aryl Acylamidase of *Bacillus sphaericus*, Catalyzing the Hydrolysis of Various Phenylamide Herbicides and Fungicides

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Received for publication 14 May 1973

From *Bacillus sphaericus* ATCC 12123 an aryl acylamidase (EC 3.5.1.13) was purified to homogeneity by ion exchange chromatography, gel filtration, and polyacrylamide gel electrophoresis. The enzyme is inducible by various phenylamides of the acylanilide, phenylcarbamate, and methoxysubstituted phenylurea type. It has a molecular weight of 75,000. Enzyme activity was inhibited by sulphydryl reagents, several metal ions, and 3,4-dichloroaniline (a product of linuron degradation). A requirement for divalent metal ions in enzyme activity could not be demonstrated. In the presence of 6 M urea an irreversible inactivation of the enzyme occurred. The hydrolysis of L-alanine-4-nitroanilide was competitively inhibited by puromycin.

In our recent reports we described the degradation of the herbicide linuron and some other herbicides and fungicides of the phenylamide type by growing cultures (32) and crude extracts obtained from linuron-induced cells of *Bacillus sphaericus* ATCC 12123 (15). The products of hydrolysis of linuron were identified as 3,4-dichloroaniline, CO<sub>2</sub>, and *N,O*-dimethylhydroxylamine (16). The acylanilides, which included herbicidal and fungicidal compounds, were hydrolyzed to the corresponding acids and anilines. On the basis of the substrate specificity, the linuron-induced enzyme was classified as an acylamidase.

These results are in contrast to the reports of Geissbühler et al. (18) and Tweedy et al. (29), who demonstrated a stepwise demethylation or demethoxylation, deamination, and decarboxylation in the microbial degradation of phenylurea herbicides. The linuron-induced *B. sphaericus* enzyme, however, does resemble the enzyme of *Pseudomonas striata* (19), which degrades phenylcarbamate and acylanilide herbicides, and the acylanilide-hydrolyzing enzymes from *Fusarium solani* (20) and *Penicillium* sp. (26). The purification and properties of a rice aryl acylamidase, which could hydrolyze 3',4'-dichloropropionanilide, have also been reported (17).

The utilization of aminoacyl- or acyl-arylamides by bacteria (5, 6, 11), fungi (13, 28), human, and animal tissues (9, 22, 23) has been a subject of investigations in many laboratories. These substrates are hydrolyzed by arylamidases (or naphthylamidases) to yield the corresponding acid and arylamine.

This report deals with the culture conditions of *Bacillus sphaericus* in respect to phenylamide metabolism and aryl acylamidase production and with the purification, properties, and specificity of this inducible enzyme.

## MATERIALS AND METHODS

**Chemicals.** <sup>14</sup>C-Ureido labeled and unlabeled 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea (linuron) were purchased from Hoechst, Frankfurt, Germany. 3',4'-Dichloropropionanilide (propanil) was kindly provided by Monsanto, St. Louis, Mo. 3',4'-dichloro-2-methacrylanilide (dicryl), 3',4'-dichloro-2-methylvalerianilide (karsil), and 3'-chloro-2'-methyl-*p*-valerotoluidide (solan) were provided by Niagara, Middleport, N.Y. 2,3-Dihydro-5-carboxanilido-6-methyl-1,4-oxathiin (carboxin) was supplied by Uniroyal, Naugatuck, Conn. 2-Methylbenzanilide, 2-chlorobenzanilide, 2,5-dimethylfuran-3-carboxanilide, and isopropylcarbanilate (propham) were isolated from commercial sources as described previously (30). 2-Methyl-5,6-dihydro-4-H-pyran-3-carboxanilide (pyracarbolid) was a gift from Hoechst,

Frankfurt, Germany; 3-(3-chloro-4-bromophenyl)-1-methoxy-1-methylurea (maloran) was from Ciba-Geigy, Basel, Switzerland; and 4-chloro- $\alpha$ , $\alpha$ -dimethylvalerianilide (monalide) was from Schering, Berlin, Germany, 4-Nitroacetanilide was purchased from EGA Chemie, Steinheim, Germany, and the aminoacyl-4-nitroanilides were products of Merck, Darmstadt, Germany. The enzymes used to calibrate a column of G-100 and G-200 Sephadex were purchased from Boehringer, Mannheim, Germany.

**Organisms and growth conditions.** Linuron degradation was examined in *Bacillus sphaericus* 150 isolated from soil and the *B. sphaericus* ATCC strains 7054, 10208, 12123, 12300, and 14577, which were kindly provided by O. Kandler, Botanical Institute, University of Munich. The bacteria were grown with shaking at 30 C in standard I medium (Merck, Darmstadt, Germany), carboxylic acid medium (1), and minimal medium (30). The carboxylic acid medium (pH 7.2) consisted of 2.5 g of di-ammonium hydrogencitrate, 0.2 g of  $\text{KH}_2\text{PO}_4$ , 0.3 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 3.0 g of sodium succinate, 4.0 g of monosodium glutamate, 20 mg of  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 1 g of yeast extract, 1 liter of distilled water, and 1 ml of a Hoagland trace element solution.

For large-scale preparations of cells, the bacteria were grown by shaking in 1-liter Fernbach vessels containing 600 ml of medium or in 10-liter carboys with 6 liters of culture aerated with a sterile stream of air.

**Degradation of fungicides and herbicides in growing cultures.** Cultivation of the bacteria for all studies on pesticide degradation was performed in 100-ml Erlenmeyer flasks containing 30 ml of medium on a shaker at 30 C. The pesticide concentration was 0.05  $\mu\text{mol/ml}$ . Because of the relatively low solubility of the compounds, the solubilizer, acetone-propylen-glycol (1:1), was used for the preparation of stock solutions of different substrates, from which the needed amounts of substrate were slowly added to the media (usually 5  $\mu\text{liters/ml}$  of culture medium).

Growth of the bacteria was determined by measuring the optical density of the cultures at 578 nm with an Eppendorf photometer. The degradation rates of the pesticides were determined by quantitative residue analysis of the compounds in the culture solution (30, 32).

**Aryl acylamidase assay.** The activity of the enzyme was determined with  $^{14}\text{C}$ -ureido-labeled linuron and L-alanine-4-nitroanilide as substrates. The assay with linuron was described previously (31). The assay with L-alanine-4-nitroanilide and other nitroanilides was performed at 30 C in tubes containing 1 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5), 1 mM substrate, and 0.001 to 0.1 ml of crude extract or corresponding fractions from enzyme purification, respectively. Reactions were terminated by the addition of 0.1 ml of 10% trichloroacetic acid, and the resulting precipitate was removed by centrifugation. The extent of hydrolysis of the compound was calculated from the release of 4-nitroaniline, measured at 405 nm ( $\epsilon = 9,620$ ).

Because of its ease and speed of performance, this assay was used to detect the enzyme in column effluents and different fractions during enzyme purifi-

cation. It was also used in some determinations of the properties of the enzyme.

Specific activity of the enzyme preparations was calculated as micromoles of substrate degraded per minute (1 U) per milligram of protein. Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as standard.

**Determination of substrate specificity.** For the determination of enzyme activity with other herbicides and fungicides, 750 nmol of each compound was added to 15 ml of 0.1 M phosphate buffer (pH 7.5) and incubated 1 to 20 h with 0.1 ml of crude extract at 30 C. The degradation rates were determined by quantitative residue analysis of the substrate in the incubation mixture (30, 32).

**Polyacrylamide gel electrophoresis.** Analytical polyacrylamide gel electrophoresis was performed at pH 8.3 with 7.5% cross-linked gels in a DESAGA vertical-plate electrophoresis apparatus by the method of Ornstein (24). Protein was detected by staining the gels with 0.25% Coomassie brilliant blue in methanol-acetic acid-water (5:1:5) for 2 h at room temperature. The gels were destained in a DESAGA destaining chamber with 5% methanol in 7% acetic acid. Activity staining of the gels with linuron as substrate was performed as described recently (15).

Preparative polyacrylamide gel electrophoresis was performed by the same method in an apparatus described by Strauch (27).

**Purification of the aryl acylamidase.** All steps of enzyme purification were carried out at 0 to 4 C. The specific activities of the various fractions were determined with both ureido- $^{14}\text{C}$ -labeled linuron and L-alanine-4-nitroanilide as substrates.

**Step 1, preparation of crude extract.** To induce the formation of the phenylamide-degrading enzyme, *B. sphaericus* ATCC 12123 was grown for 48 h in minimal medium in the presence of 0.05  $\mu\text{mol}$  of linuron per ml. A 60-g (wet weight) batch of cells was washed three times with cold 0.05 M phosphate buffer pH 7.5 and ground with alumina (Martinswerk, Bergheim, Erft, type PFT) in a chilled mortar. The resulting paste was extracted with 100 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 0.1 mM dithioerythrol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 20% (vol/vol) glycerol for stabilization of the enzyme. The extract was first centrifuged at  $40,000 \times g$  for 10 min. The pellet was discarded, and the supernatant fluid was subjected to centrifugation at  $100,000 \times g$  for 60 min. The resulting supernatant fluid was used as crude extract.

**Step 2, protamine sulfate precipitation.** To the crude extract (104 ml), 26 ml of 1.5% protamine sulfate in 0.05 M Tris-hydrochloride buffer (pH 7.5) was slowly added with stirring to give a final concentration of 0.3%. After 5 min the precipitate was removed by centrifugation at  $20,000 \times g$  for 10 min and discarded.

**Step 3, calcium phosphate gel adsorption.** To the supernatant fraction from the previous step, 18 g of freshly prepared calcium phosphate gel (12) was added. The mixture was stirred for 10 min and centrifuged at  $20,000 \times g$  for 10 min. In this step most of the enzyme remained in the supernatant fluid, and the precipitate was discarded.

**Step 4, DEAE-cellulose chromatography.** The enzyme was further purified by chromatography on DEAE-cellulose (Whatman DE 52). The cellulose was equilibrated in 0.05 M KCl in 0.05 M phosphate buffer (pH 7.5) containing 0.1 mM dithioerythrol and 1 mM EDTA and packed into a column (30 by 300 mm). The enzyme solution from step 3 was dialyzed against buffer and applied to the column. The column was first washed with 650 ml of buffer which removed about 30% of the initially applied protein. The enzyme was then eluted from the column between 0.18 and 0.22 M KCl after application of a linear gradient formed with 500 ml of buffer and 500 ml of elution buffer with 0.4 M KCl. Elution was carried out at a flow rate of 0.6 ml/min, and 6.2-ml fractions were collected. The fractions containing the enzyme were combined, and the solution was concentrated by ultrafiltration in an Amicon Diaflo cell with a UM-10 membrane.

**Step 5, gel filtration on G-100 and G-200 Sephadex.** The concentrated enzyme solution from step 4 was dialyzed against 0.05 M Tris-hydrochloride buffer (pH 7.5) with addition of 0.1 mM dithioerythrol, 1 mM EDTA, and 20% (vol/vol) glycerol and was then applied to a Sephadex column. The column consisted of two layers of Sephadex. The lower part (50 by 300 mm) was filled with G-200 Sephadex, and the upper part (50 by 500 mm) was filled with G-100 Sephadex equilibrated in the Tris buffer given above. The column was run at 0.3 ml/min, and fractions of 2.8 ml were collected. Fractions containing the enzyme were combined, and the volume was reduced by ultrafiltration as described above.

**Step 6, gel filtration on G-200 Sephadex.** The concentrated fraction from the previous step was applied onto a column (50 by 800 mm) packed with G-200 Sephadex, and fractionated under the same conditions as described above. Active fractions from the second gel filtration procedure were again combined and concentrated as described above.

Examination of this fraction by polyacrylamide gel electrophoresis showed the presence of one major and three to four minor protein bands. Activity staining showed that the enzyme was associated with the major protein band.

**Step 7, preparative polyacrylamide gel electrophoresis.** Final purification of *B. sphaericus* aryl acylamidase was obtained by preparative polyacrylamide gel electrophoresis in an apparatus described by Strauch (27), under the same conditions as used for analytical gel electrophoresis (24). The 8-cm polyacrylamide gel was eluted at a flow rate of 0.6 ml/min with 0.05 M Tris-hydrochloride buffer (pH 7.5) with addition of 0.1 mM dithioerythrol and 1 mM EDTA (Fig. 1). The active fractions (1.9 ml) were combined and concentrated as described. Final examination of the preparation by analytical gel electrophoresis showed the presence of a single protein band which was enzymatically active.

## RESULTS

**Degradation of linuron and growth of *B. sphaericus* in different culture media.** To find the best starting material for the preparation of the *B. sphaericus* aryl acylamidase, the distri-

bution of the enzyme among various *B. sphaericus* strains was first studied, and the optimal conditions for the induction of the enzyme were investigated. Therefore six strains of *B. sphaericus* were examined for growth and linuron-degrading activity in three different media. Growth of bacteria and herbicide degradation were followed daily over a period of 1 week. Degradation rates were determined by quantitative residue analysis (30, 32). In all samples the degradation of the herbicide started in the late exponential phase of growth. According to the results of Wallnöfer et al. (31), *B. sphaericus* ATCC 12123 and the strain isolated from soil (isolate 150) had the highest degradation activities in each medium examined, whereas the ATCC strains 10208, 14577, 7054, and 12300 showed considerably lower activity. Maximal degradation rates were obtained with strain 12123 and the soil-isolate after growth in minimal medium (Table 1). Since these two strains showed nearly identical degradation rates, all further experiments were performed with strain 12123.

**Specific activity of crude extracts from *B. sphaericus* ATCC 12123 after growth in different media.** To compare more precisely the ability of strain 12123 to degrade linuron after growth in the three different media, the specific activity of crude extracts from cells grown for 48 h in the media in the presence of 0.05  $\mu$ mol of linuron per liter was determined. The specific activity of the crude extract from cells grown in minimal medium was more than 50 times higher than that of the two other extracts (Table 2).

**Formation of the *B. sphaericus* aryl acylamidase during growth of a culture.** As described previously (15), the formation of the phenylamide-degrading aryl acylamidase in *B. sphaericus* is induced by the substrate linuron. In a further experiment, the formation of this enzyme during growth of a culture in the presence of 0.05  $\mu$ mol of linuron per ml was studied. Cells were harvested after 5, 6, 8, 12, 24, and 48 h of growth, and the media were tested for residual herbicide. From washed cells, crude extracts were prepared and tested for linuron-degrading activity. The specific activity of crude extracts as compared with the growth phase of the cells and degradation of linuron during growth is shown in Fig. 2. As seen from this figure, formation of the enzyme is initiated in the late exponential phase of growth. After 12 h, at the beginning of the stationary phase of growth, maximal enzyme formation is obtained. Specific activity of crude extracts from cells harvested at different times during the station-

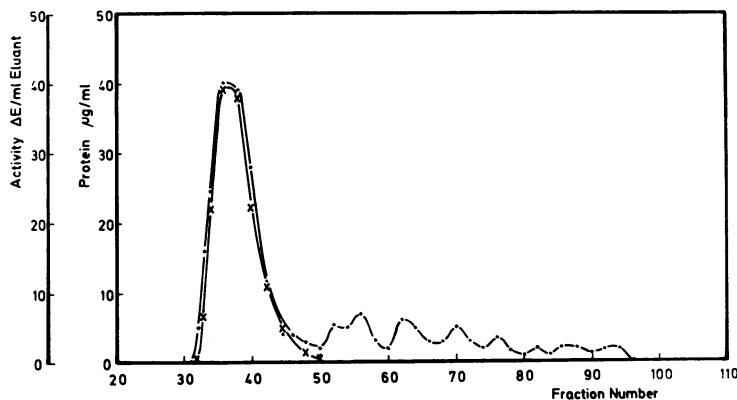


FIG. 1. Preparative polyacrylamide gel electrophoresis of fraction 6. Symbols: ×, aryl acylamidase activity; ●, protein.

TABLE 1. Percent degradation of linuron (0.05  $\mu\text{mol/ml}$ ) by different strains of *B. sphaericus* after 1 and 2 days of growth in standard I medium, carboxylic acid medium (1), and minimal medium (30)

| Strain      | Degradation of linuron (%) |        |                        |        |                |        |
|-------------|----------------------------|--------|------------------------|--------|----------------|--------|
|             | Standard I-medium          |        | Carboxylic acid medium |        | Minimal medium |        |
|             | 1 Day                      | 2 Days | 1 Day                  | 2 Days | 1 Day          | 2 Days |
| Isolate 150 | 6                          | 52     | 9                      | 50     | 55             | 100    |
| ATCC 12123  | 0.0                        | 22     | 27                     | 66     | 95             | 100    |
| ATCC 10208  | 0.0                        | 32     | 0.0                    | 40     | 0.0            | 0.0    |
| ATCC 12300  | 0.0                        | 0.0    | 0.0                    | 16     | 4              | 10     |
| ATCC 7054   | 4                          | 30     | 6                      | 18     | 6              | 10     |
| ATCC 14577  | 8                          | 24     | 0.0                    | 19     | 5              | 10     |

TABLE 2. Degradation of linuron and specific activity of crude extracts of *Bacillus sphaericus* after growth in different media

| Culture medium         | Optical density after growth for 2 days (578 nm) | Degradation of linuron after 2 days (%) | Cell yield (g dry weight/liter) | Sp act of crude extract ( $\text{U} \times 10^{-3}/\text{mg}$ of protein) |
|------------------------|--|---|---------------------------------|---|
| Standard I medium      | 0.95   | 25                                      | 1.5                             | 0.002   |
| Carboxylic acid medium | 1.15   | 60                                      | 2.9                             | 0.002   |
| Minimal medium         | 0.34   | 100                                     | 0.33                            | 0.13  |

ary phase of growth then remains constant, although degradation of the herbicide was complete after 24 h.

**Induction of *B. sphaericus* aryl acylamidase by other phenylamides.** The linuron-induced aryl acylamidase of *B. sphaericus* degrades a large variety of herbicides and fungi-

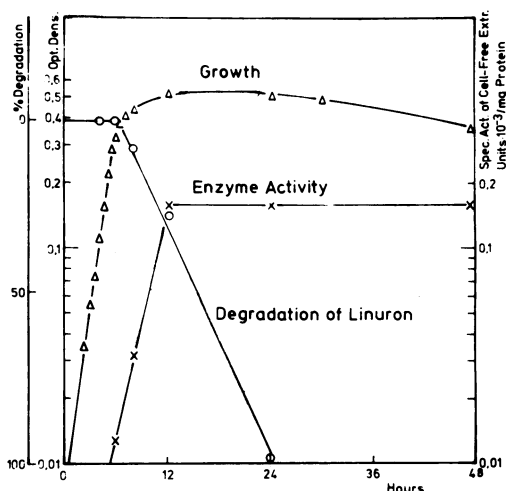


FIG. 2. Time course of linuron degradation and formation of the linuron-degrading aryl acylamidase during growth of *B. sphaericus* ATCC 12123 in minimal medium.

cides of the phenylamide type as described previously (15). It was tested whether some of the substrates could also serve as inducers of the enzyme. *B. sphaericus* was cultivated in minimal medium in the presence of 0.05  $\mu\text{mol/ml}$  of the phenylurea herbicide maloran, the acylamide herbicides monalide and propanil, the acylanilide fungicides 2-chlorobenzanilide and 2,5-dimethyl-furan-3-carboxanilide, and the phenylcarbamate herbicide prophan. After 2 days the cells were harvested and crude extracts were prepared and examined for enzyme activity. All compounds tested were able to induce the formation of the *B. sphaericus* aryl acylamidase (Table 3). The specific activity of the various extracts, however, was considerably lower than that of crude extracts from linuron-induced cells.

**Purification of the *B. sphaericus* aryl acylamidase.** With the procedure described above, a purification of the enzyme to homogeneity as judged by polyacrylamide gel electrophoresis was obtained. The purification was 73-fold with a yield of 2%. The data of a typical purification are summarized in Table 4.

**Properties of the enzyme.** Since the enzyme proved to be very labile during large-scale preparation, only partially purified preparations, usually those from step 3 or 4, were used for enzyme characterization.

**Influence of pH and different buffers.** The pH optimum for the aryl acylamidase activity has been already determined by Wallnöfer et al. (31), who observed a broad pH optimum from pH 7.0 to 8.5. Enzyme activity was the same in both 0.1 M Tris-hydrochloride (pH 7.5) and 0.1 M phosphate (pH 7.5), with linuron and L-alanine-4-nitroanilide as substrates.

TABLE 3. Comparison of aryl acylamidase activity of crude extracts obtained from *B. sphaericus* cells grown in the presence of different phenylamides

| Inducer   | Sp act of crude extracts (U × 10 <sup>-2</sup> /mg of protein) | Rate of induction compared with extracts from linuron-induced cells (%) |
|---|--|---|
| 3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea (linuron)         | 0.13   | 100   |
| 3-(3-Chloro-4-bromophenyl)-1-methoxy-1-methylurea (maloran)     | 0.015  | 12  |
| 4-Chloro- $\alpha$ , $\alpha$ -dimethylvalerianilide (monalide) | 0.029  | 22  |
| 3',4'-Dichloropropionanilide (propanil)                         | 0.002  | 2   |
| 2-Chlorobenzanilide   | 0.03   | 23  |
| 2,5-Dimethylfuran-3-carboxanilide                               | 0.05   | 40  |
| Isopropylcarbanilate (propham)                                  | 0.008  | 6   |

**Influence of various inhibitors and effect of metal ions.** A variety of compounds was tested for possible effects on the degradation of ureido-<sup>14</sup>C-labeled linuron. Before the addition of the substrate, all samples were preincubated in the presence of the inhibitor for 15 min. Degradation of linuron was slightly inhibited by the two sulfhydryl reagents sodium arsenate and iodoacetamide (IAA) (Fig. 3). *p*-Chloromercuribenzoate was a more toxic inhibitor. A 10<sup>-5</sup> M concentration of this compound caused a 50% reduction of enzyme activity, and 10<sup>-4</sup> M caused a 75% inhibition, respectively. Furthermore, the enzyme was sensitive to Cu<sup>2+</sup>, Zn<sup>2+</sup>, Ag<sup>+</sup>, and particularly Hg<sup>2+</sup> ions. At 10<sup>-4</sup> M, Ag<sup>+</sup> caused a 6%, Cu<sup>2+</sup> a 9.5%, Zn<sup>2+</sup> a 51%, and Hg<sup>2+</sup> a 98% reduction of enzyme activity. These results indicate that sulfhydryl groups of the enzyme are presumably involved in enzyme activity. The presence of 10<sup>-3</sup> M Co<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>2+</sup>, Pb<sup>2+</sup>, Mg<sup>2+</sup>, and Mn<sup>2+</sup> ions caused neither inhibition nor activation of the enzyme. Among the chelating agents tested (potassium

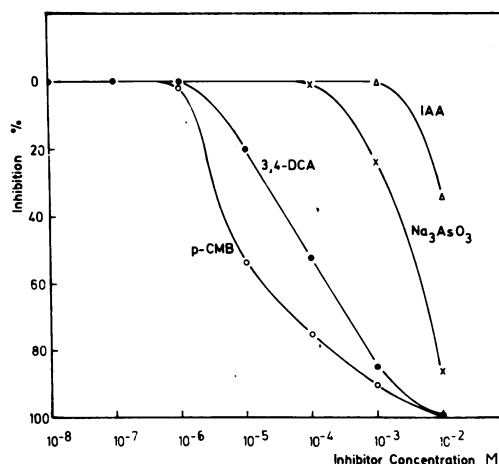


FIG. 3. Inhibition of enzyme activity by the sulfhydryl reagents *p*-chloromercuribenzoate (*p*-CMB), iodoacetamide (IAA), and sodium arsenate (Na<sub>3</sub>AsO<sub>3</sub>) and by 3,4-dichloroaniline (3,4-DCA).

TABLE 4. Summary of aryl acylamidase purification procedure

| Fraction                                  | Protein (mg) | Total enzyme activity (U × 10 <sup>-2</sup> ) | Sp act (U × 10 <sup>-2</sup> /mg of protein) | Recovery (%) |
|---|--------------|---|--|--------------|
| 1. Crude extract                          | 2,370        | 284   | 0.12   | 100          |
| 2. Protamine sulfate precipitation        | 1,475        | 221   | 0.15   | 77.7         |
| 3. Calcium phosphate gel adsorption       | 608          | 201   | 0.33   | 70.6         |
| 4. DEAE-cellulose chromatography          | 67.2         | 106   | 1.58   | 37.3         |
| 5. Gel filtration on G-100/G-200 Sephadex | 15.7         | 46.6  | 2.97   | 16.4         |
| 6. Gel filtration on G-200 Sephadex       | 3.28         | 19.0  | 5.78   | 6.7          |
| 7. Polyacrylamide gel electrophoresis     | 0.67         | 5.8   | 8.72   | 2.0          |

cyanide, sodium sulfide, sodium azide, and EDTA) only sodium sulfide at a concentration of 1 mM inhibited the enzyme activity. Dialysis of the enzyme for 20 h against 1 mM EDTA also did not affect enzyme activity. Thus, this aryl acylamidase lacks a demonstrable metal ion requirement, unless the failure of inhibition by EDTA reflects an extremely firm binding of metal ions.

The end product of linuron degradation, 3,4-dichloroaniline (3,4-DCA), did affect the degradation of linuron by crude extracts. In 10  $\mu$ M concentration, this compound still caused a 20% inhibition of enzyme activity (Fig. 3). The incubation of the crude extract in 6 M urea in 0.1 M Tris-hydrochloride buffer (pH 7.5) resulted in an irreversible inactivation of the enzyme.

**Effect of puromycin and penicillin G on enzyme activity.** In their studies on different arylamidases Behal et al. reported that puromycin was a competitive inhibitor of *Neisseria catarrhalis* arylamidase (7, 8) and aminopeptidases from human tissue (4, 9). The *N. catarrhalis* arylamidase had no penicillin G amidase activity. In another report, Ellis and Nuenke (14) described a strong inhibition of a dipeptidyl arylamidase of the pituitary by puromycin.

The influence of puromycin and penicillin G on the *B. sphaericus* aryl acylamidase was determined with L-alanine-4-nitroanilide as substrate. L-Alanine-4-nitroanilide concentration varied from 0.01 to 1 mM; inhibitor concentrations were 0.0, 0.1, and 0.2 mM with puromycin and 0.0, 0.01, 0.1, 1, and 2 mM with penicillin, respectively. In contrast to penicillin G, which did not influence the hydrolysis of L-alanine-4-nitroanilide, puromycin inhibited the degradation of this compound competitively (Fig. 4). Further experiments were then made as to whether penicillin G or puromycin might serve as substrates of the enzyme. However, no degradation products of these two antibiotics could be detected.

**Temperature stability.** During storage of crude extracts at  $-15^{\circ}\text{C}$  for 3 months, no loss of activity could be detected. The purified enzyme lost no activity after 12 weeks storage at the same temperature. After 12 months, 50% of the initial activity was still present.

For determination of heat inactivation, a partially purified enzyme was incubated for 1 to 20 min at temperatures between 20 and 50  $^{\circ}\text{C}$ . After incubation the activity was determined with L-alanine-4-nitroanilide as substrate. The results showed that the enzyme was inactivated to 50% within 9.5 min at 40  $^{\circ}\text{C}$ , within 2 min at 45  $^{\circ}\text{C}$ , and within 0.5 min at 50  $^{\circ}\text{C}$ .

**Molecular weight of the enzyme.** The molecular weight of the enzyme was estimated by a modification of the gel filtration method of Andrews (2). The same column (packed with G-100 and G-200 Sephadex) which was used for enzyme purification was used for molecular weight determination. To calibrate the column cytochrome c (molecular weight 13,000), pig heart malic dehydrogenase (molecular weight 40,000), and calf alkaline phosphatase (molecular weight 100,000) were used as reference proteins. A sample containing 0.2 mg of cytochrome c, 0.5 mg of malic dehydrogenase, 0.1 mg of alkaline phosphatase, and 11.5 mg of partially purified enzyme from *B. sphaericus* (fraction 3) was applied to the column, which was washed with 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 0.1 mM dithioerythrol, 1 mM EDTA, and 20% glycerol at a flow rate of 0.3 ml/min. Fractions of 17.5 ml were collected. According to the elution pattern (Fig. 5), the molecular weight of aryl acylamidase is  $75,000 \pm 10\%$ .

**Substrate specificity.** The linuron-induced aryl acylamidase from *B. sphaericus* catalyzes the degradation of a large variety of different herbicides, fungicides, and other substrates of the phenylamide type (15, 32). In addition to the substrates previously described, the acylanilide herbicides propanil, karsil, dicryl and solan, as well as the phenylcarbamate protham, were also degraded to their corresponding anilines by crude extract of *B. sphaericus* grown in

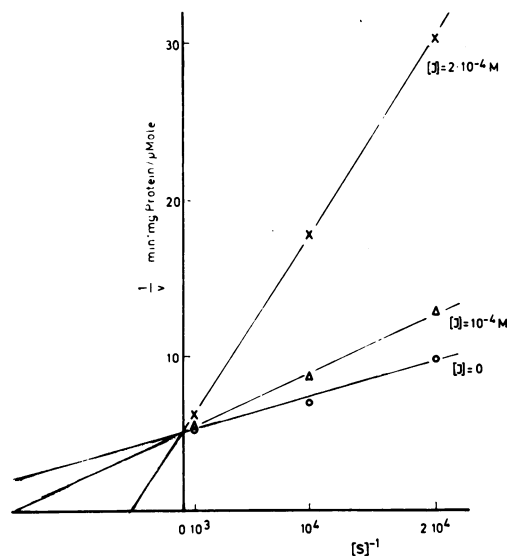


FIG. 4. Competitive inhibition of aryl acylamidase by puromycin.

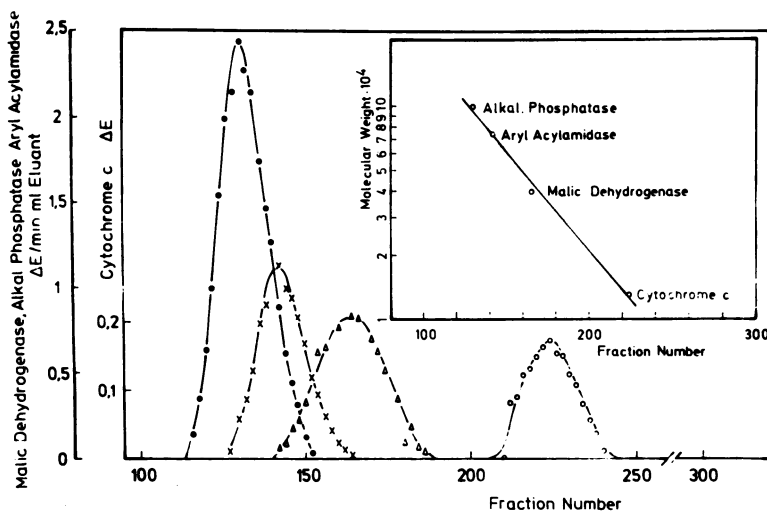


FIG. 5. Determination of the molecular weight of *B. sphaericus* aryl acylamidase (x) by gel filtration. Reference proteins are: cytochrome c (measured at 405 nm; ○); alkaline phosphatase (measured as described by Bergmeyer (10; ●); malic dehydrogenase (measured as described by Wolf (33; Δ).

the presence of linuron at rates of 249, 194, 144, 222, and  $9.9 \text{ U} \times 10^{-3}$  per mg of protein, respectively. It should be mentioned again that only crude extracts obtained from linuron-induced cells were active in degrading these compounds; crude extracts from noninduced cells did not show any activity towards the different phenylamides used. The aryl acylamidase catalyzed hydrolysis of the various phenylamides was linear with respect to the amount of crude extract added (Fig. 6 and 7). The time dependence of deacylation of L-alanine-4-nitroanilide, N-acetyl-L-alanine-4-nitroanilide and 2,5-dimethylfuran-3-carboxanilide is shown in Fig. 8.

From the products formed during the hydrolysis of the phenylamides and from the fact that the enzyme showed the highest specific activities with simple acylanilides, it was concluded that the amide bond between the aniline moiety and the carboxy group is the site of enzyme attack even for the phenylurea and the phenylcarbamate herbicides and the enzyme was thus classified as an aryl acylamidase (EC 3.5.1.13, aryl-acylamide amidohydrolase). In view of these results a degradation scheme as shown in Fig. 9 is proposed.

**Michaelis constants and turnover numbers of aryl acylamidase with different substrates.** Michaelis constants were calculated for different substrates according to the method of Lineweaver and Burk (Fig. 10).

The  $K_m$  values for L-alanine-4-nitroanilide and L-leucine-4-nitroanilide were  $9 \times 10^{-5}$  and  $2 \times 10^{-4}$  M; and for linuron and 2,5-dimethylfuran-3-carboxanilide,  $2 \times 10^{-6}$  and  $3 \times 10^{-5}$  M,

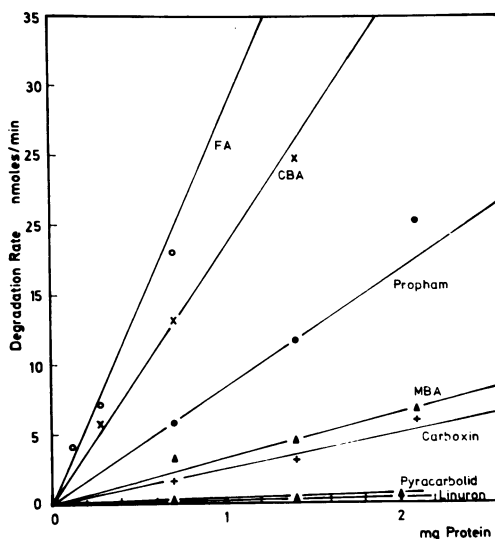


FIG. 6. Influence of enzyme concentration on degradation of different herbicides and fungicides. The tests were performed with crude extract obtained from linuron-induced cells.

respectively. These data indicate that the linuron-induced aryl acylamidase shows the highest affinity to its inducer linuron, followed by 2,5-dimethylfuran-3-carboxanilide, L-alanine-4-nitroanilide, and L-leucine-4-nitroanilide with decreasing affinity for the enzyme.

From the specific activity of the purified enzyme with linuron and L-alanine-4-nitroanilide as substrates and the molecular weight, the turnover numbers of the two substrates were calculated as 1,029 for L-alanine-4-nitroanilide

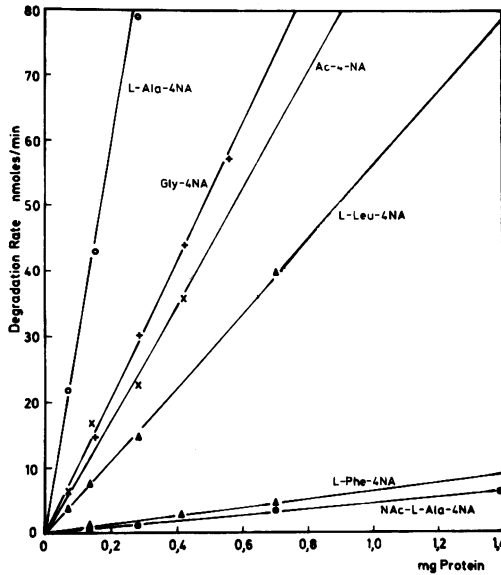


FIG. 7. Influence of enzyme concentration on degradation of different acyl-4-nitroanilides by crude extract from linuron-induced cells.

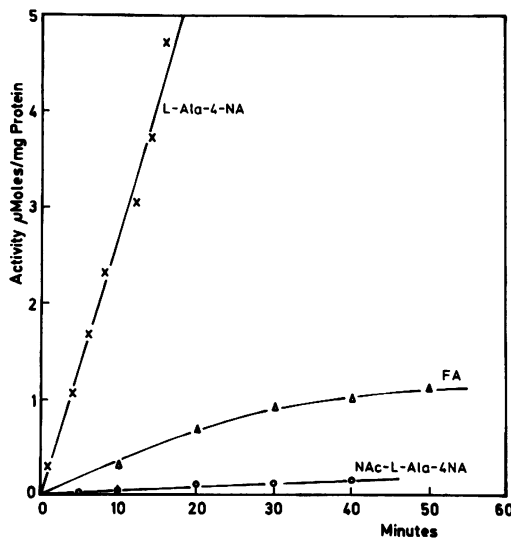


FIG. 8. Influence of time on degradation of *L*-alanine-4-nitroanilide (*L*-Ala-4NA), *N*-acetyl-*L*-alanine-4-nitroanilide (*N*Ac-*L*-Ala-4NA), and 2,5-dimethylfuran-3-carboxanilide (FA) by crude extract from linuron-induced cells.

and 0.65  $\mu\text{mol}$  of substrate per  $\mu\text{mol}$  of enzyme for linuron.

## DISCUSSION

Comparison of the *Bacillus sphaericus* aryl acylamidase with other aryl- or naphthylamidases described in the literature reveals various common properties. The inducibility of the aryl

acylamidases from *Penicillium* sp. and *Fusarium solani* by karsil or propanil, respectively, was demonstrated by Sharabi and Bordeleau (26) and Lanzilotta and Pramer (20). Aubert and Millet (3) found that the *L*-leucyl- $\beta$ -naphthylamide hydrolase in *B. megaterium* is more repressed during growth than during sporulation. A relationship between sporulation and inducibility of the aryl acylamidase is also observed in *B. sphaericus*. The enzyme is formed during the late exponential and early stationary phase of growth, although linuron is present during the growth of the cells for at least three generations (Fig. 2). When cultures were examined by phase contrast microscopy, it was found that those cultures which displayed a high rate of linuron degradation contained more spores than others with a low rate of linuron degradation. Thus, in *B. sphaericus* ATCC 12123 after growth in minimal medium, approximately 25% of the cells had sporulated, whereas after growth in standard I and carboxylic acid medium no more than 2 to 3% of the cells had sporulated. Examination of the different strains

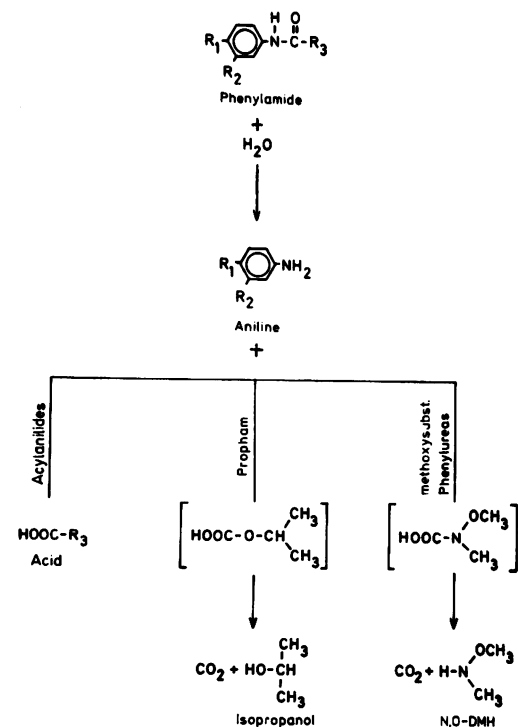


FIG. 9. Proposed mechanism for the degradation of phenylamides by the *B. sphaericus* aryl acylamidase;  $R_2$ : -H, -Cl;  $R_1$ : -H, -Cl, -Br, -CH<sub>3</sub>, -NO<sub>2</sub>;  $R_3$ : alkyl group (acyl- and aminoacylanilides), alkoxy group (prophan), methoxymethylamino group (phenylureas).



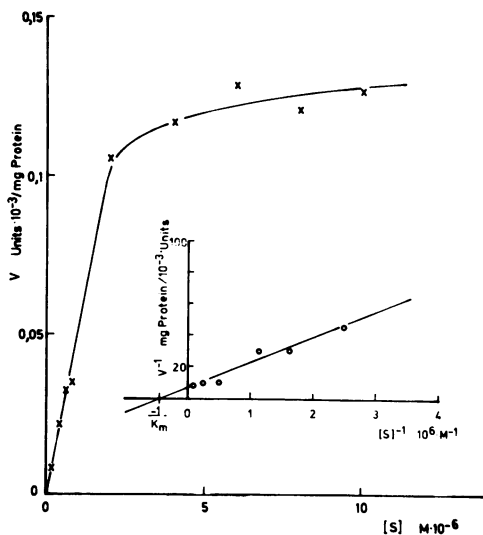


FIG. 10. Determination of  $K_m$  for linuron as substrate.

of *B. sphaericus* (Table 1) revealed a similar relationship in that those strains having a high rate of linuron degradation showed also some sporulation.

The *B. sphaericus* aryl acylamidase was quite sensitive to inhibition by sulfhydryl reagents and different metal ions and had no metal ion requirement for enzyme activity. The hydrolysis of L-alanine-4-nitroanilide was competitively inhibited by puromycin. In their studies on the relationship between alanyl-glycine dipeptidase and arylamidase of *Neisseria catarrhalis*, Behal and Folds (8) reported that the dipeptidase required  $Mn^{2+}$  or  $Mg^{2+}$  ions for stability and was inhibited by chelating agents and *p*-chloromercuribenzoate, but not by puromycin. For the arylamidase no metal ion requirement could be demonstrated and the enzyme was strongly inhibited by *p*-chloromercuribenzoate and puromycin, whereas EDTA was not inhibitory. Inhibition by puromycin was competitive (7). Furthermore, the enzyme was strongly inhibited by  $Zn^{2+}$ ,  $Cu^{2+}$  and  $Hg^{2+}$  ions. Similar properties were also described by other authors for the arylamide hydrolyzing enzymes of *Pseudomonas aeruginosa* (25), chick kidney (23), rice (17), and rat liver and kidney (22). Most bacterial enzymes do not require metal ions for activity (7, 11), whereas the arylamidase of *P. aeruginosa* (25) requires  $Mn^{2+}$  or  $Mg^{2+}$  ions, the naphthylamidase from Baker's yeast (28) requires  $Ba^{2+}$ , and the mammalian arylamidases (4, 22) require  $Co^{2+}$ ,  $Mn^{2+}$ ,  $Ni^{2+}$ , or  $Zn^{2+}$  ions for optimal activity.

For the *B. sphaericus* aryl acylamidase, a molecular weight of approximately 75,000 was

determined, which is one half of the value reported for the enzyme from *N. catarrhalis* (6). However, it is quite close to the values determined for the arylamidases of *Sarcina lutea* (5) and *P. aeruginosa* (25).

The substrate specificity of the *B. sphaericus* enzyme was shown to be rather low in that acylanilide, phenylcarbamate, and methoxy-substituted phenylurea compounds are hydrolyzed. The herbicide-degrading enzymes from *Pseudomonas striata* (19), *F. solani* (20), and *Penicillium* sp. (26) had considerably higher substrate specificities. The highest activity of the *B. sphaericus* enzyme was obtained with L-alanine-4-nitroanilide as substrate. A similar specificity for L-alanine- $\beta$ -naphthylamide was also found for the enzymes of *N. catarrhalis* (7), *P. aeruginosa* (25), pathogen strains of *Leptospira* (11), and mammalian tissue (4).

Although a number of aryl acylamidases have been studied, the role of these enzymes in microbial metabolism remains obscure. It is of great interest, however, that these enzymes are inducible by some herbicides and fungicides and are able to degrade these compounds.

#### ACKNOWLEDGMENTS

This investigation was supported by a grant from the Deutsche Forschungsgemeinschaft.

We gratefully acknowledge the valuable technical assistance of Beate Schumacher.

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