APPLIED MICROBIOLOGY, May 1970, p. 714-717

Vol. 19, No. 5
Printed in U.S.A.

Degradation of Urea Herbicides by Cell-Free Extracts of *Bacillus sphaericus*

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Received for publication 2 January 1970

N'-methoxy phenylurea herbicides are degraded by induced cells of *Bacillus sphaericus* ATCC 12123 by liberating carbon dioxide from the ureido portion of the molecule and leaving the corresponding aniline moieties. Cell-free extracts of *B. sphaericus* inactivate these herbicides in the same way as reported for whole cells. A 6.6-fold purification of the crude extract was achieved by a combination of salt fractionation with ammonium sulfate and column chromatography on diethylaminoethyl cellulose.

Substituted urea derivatives constitute a large class of compounds which show herbicidal activity. Previous reports indicate that the degradation of *N'*-methoxy phenylurea herbicides occurs rapidly in soils, and that soil microorganisms are mostly responsible for its breakdown (1).

The isolation and identification of a soil bacterium, *Bacillus sphaericus*, which decomposes *N'*-methoxy phenylurea herbicides have been reported (5). The microorganism inactivates the herbicides by liberating carbon dioxide from the ureido portion of the molecule and leaving the halogen-substituted aniline moieties (6).

This report describes the results of our study on degradation of different urea herbicides by several strains of *B. sphaericus* and the partial purification and properties of an enzyme from the cell-free extract of *B. sphaericus*. This enzyme preparation was found to be responsible for the hydrolysis of these urea compounds.

MATERIALS AND METHODS

Strains. A *B. sphaericus* strain isolated from soil (5) and ATCC strains 14577, 12300, 12123, 10208, 7055, and 7054 (provided by O. Kandler, Botanical Institute, University of Munich) were used in this study. The bacteria were grown in 6 liters of a medium containing 0.1% yeast extract as described previously (6), but the vitamin mixture was omitted. To induce the enzyme system, bacteria were grown in the presence of 50 μmoles of linuron (Table 2). Cells were harvested in the late logarithmic-growth phase and stored at −15 C, after being washed twice with distilled water.

Herbicides. The enzymatic degradation of the substituted urea herbicides (monuron, monolinuron, linuron, metobromuron, and fluometuron) was investigated (Table 2). The preparation of the used herbicides and the residue analysis were performed as described (6). The products of the reaction were identified by thin-layer chromatography and infrared spectroscopy.

Enzymatic assays. The packed cells were ground in a mortar at 0 C with twice their weight of Al_2O_3 and then suspended in 0.1 M phosphate buffer (*p*H 7.0). The crude extract was centrifuged at 20,000 × *g* for 45 min at 4 C. All the following procedures were performed at 4 C.

Enzyme activity was measured by incubating the enzyme preparation with ureido-^{14}C-labeled linuron (specific activity, 2,600 counts per min per μmole) in 0.1 M phosphate buffer (*p*H 7.0) in sealed Erlenmeyer flasks at 30 C on a shaker. $^{14}CO_2$ was trapped in separate reaction vials containing 1.5 ml of 1 N NaOH. The $^{14}CO_2$ was determined in a liquid scintillation counter (Beckman Instruments, Fullerton, Calif.) with a counting efficiency of about 90%. No corrections, except for background, were applied to the counting data. When the other herbicides were used as substrates, enzyme activity was assayed by determining the reaction products, namely the halogen-substituted anilines (6). The reaction was stopped after 120 min by adding 0.5 ml of 10 N H_3PO_4 per 10 ml of buffer to the closed system. The carbon dioxide remaining in the reaction vessels after acidification was allowed to be absorbed by the base with 30 min of additional shaking. The specific activity is defined as micromoles of herbicide degraded per minute per milligram of protein. Protein was determined by the Lowry procedure (4).

Purification of the enzyme. Two milliliters of 2% streptomycin sulfate was added to 20 ml of cell-free extract of *B. sphaericus* (3). The precipitate collected by centrifugation was discarded. The second step of purification was achieved by salt fractionation by using ammonium sulfate from 30 to 100% saturation. The precipitates collected after centrifugation were diluted with 0.1 M phosphate buffer, *p*H 7.0 (Table 1). After dialyzing (dialysis tubing no. 4465 A2, A. T. Thomas Co.) overnight against 0.05 and 0.01 M phosphate

TABLE 1. *Purification scheme*

Purification step	Protein	Yield	Specific activity	Purification
	mg/ml	%	*units*	
Crude extract	18.2	87.5	8.5×10^{-3}	
Streptomycin sulfate	16.3	100.0	10.1×10^{-3}	1.2
Ammonium sulfate precipitation (50 to 70%)	5.3	60.8	18.9×10^{-3}	2.2
Dialysate	4.9	39.0	13.1×10^{-3}	1.5
DEAE[a] cellulose	0.28	9.5	56.5×10^{-3}	6.6

[a] Diethylaminoethyl.

buffer (*p*H 8.0) and two changes of distilled water, the specific activity of the enzyme decreased by about 30%. The desalted protein was washed onto a column (1.5 by 30 cm) of diethylaminoethyl (DEAE) cellulose with 0.05 M phosphate buffer (*p*H 8.0). DEAE cellulose was prepared by the method of Flodin (2). The column was eluted with gradient concentrations of KCl. The ionic strength of the eluent was continuously increased up to 0.6 M KCl. Fractions (8 ml) were collected automatically. Fractions containing the highest activity were combined and saturated with ammonium sulfate to 80%. The precipitate was collected by centrifugation at 20,000 × *g* for 40 min and assayed for enzymatic activity (Table 1).

RESULTS AND DISCUSSION

The *N'*-methoxy phenylurea herbicides could be degraded by cell-free extract from *B. sphaericus* only when grown in the presence of the herbicides. No measurable activity was found in uninduced bacteria. Induction, however, was possible only in the above minimal medium, but not in an enriched medium containing peptone or meat extract.

B. sphaericus ATCC 12123 proved to be as active in decomposing *N'*-methoxy phenylurea herbicides as the wild type isolated from soil. ATCC strains 7055 and 7054 showed a considerably lower rate of degradation. However, ATCC strains 14577, 12300, and 10208 were completely inactive in decomposing substituted urea herbicides. Breakdown proceeded in the same way as described for the wild type (6).

Since ATCC strain 12123 showed the highest rate of inactivation of *N'*-methoxy phenylureas, this microorganism was selected for further studies with cell-free systems.

A crude extract decomposed the *N'*-methoxy phenylurea herbicides, monolinuron, linuron, and metobromuron, in the same way as reported for whole cells. Cell envelopes and a boiled extract had no activity (Fig. 1).

The enzyme system responsible for hydrolyzing the herbicides was partially purified by salt fractionation and column chromatography on DEAE cellulose, yielding a 6.6-fold purification. The

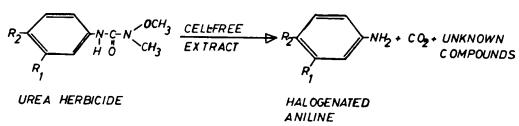

FIG. 1. *Proposed pathway for degradation of N'-methoxy phenylureas (monolinuron, R_1 = H, R_2 = Cl; linuron, R_1 = Cl, R_2 = Cl; and metobromuron, R_1 = H, R_2 = Br) by a cell-free extract of B. sphaericus ATCC 12123.*

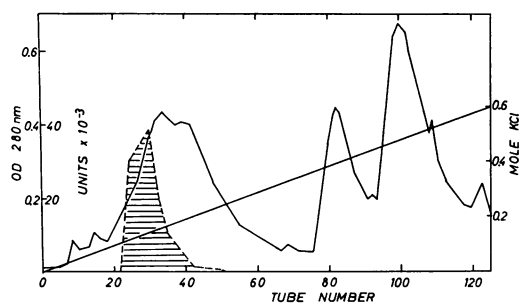

FIG. 2. *Elution pattern of cell-free extract on DEAE cellulose. Straight line indicates the elution gradient; shaded zone indicates enzyme activity.*

precipitates from 50 to 70% ammonium sulfate saturation indicated the highest enzyme activity (Table 1). Figure 2 shows the elution pattern of the enzyme from DEAE cellulose.

The optimal *p*H level of the partially purified enzyme is shown in Fig. 3. Optimal activity ranges between *p*H 7.0 and 8.5. No activity was observed at or below *p*H 5.5.

Figure 4 demonstrates that enzyme activity is linear with respect to enzyme concentrations up to 5 mg of protein.

The metabolites formed from monolinuron, linuron, and metobromuron by enzymatic hydrolysis were 4-chloroaniline, 3,4-dichloroaniline, and 4-bromoaniline, respectively.

Substrate specificity. The cell-free extract exhibited a significant substrate specificity for *N'*-methoxy phenylurea compounds, whereas

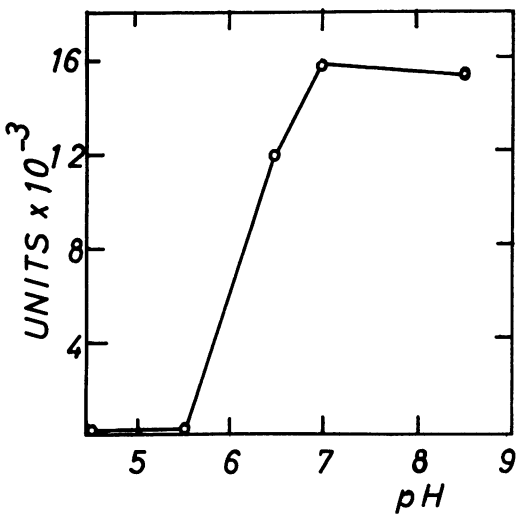

FIG. 3. *Effect of pH on enzyme activity.*

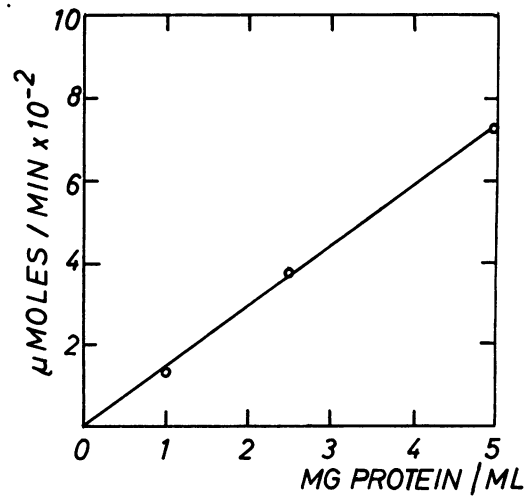

FIG. 4. *Enzyme activity as a function of the amount of protein.*

TABLE 2. *Phenylurea compound used in this study: effect of N'-methoxy group on the degradation rate of the substituted phenylurea herbicides by cell-free extract of B. sphaericus ATCC 12123*

Urea compound	Common name	Specific activity
		units
N-(4-chlorophenyl)-*N'*,*N'*-dimethylurea	Monuron	4.1×10^{-3}
N-(4-chlorophenyl)-*N'*-methoxy-*N'*-methylurea	Monolinuron	14.6×10^{-3}
N-(3,4-dichlorophenyl)-*N'*-methoxy-*N'*-methylurea	Linuron	17.8×10^{-3}
N-(4-bromophenyl)-*N'*-methoxy-*N'*-methylurea	Metobromuron	16.2×10^{-3}
N-(3-trifluoromethylphenyl)-*N'*,*N'*-dimethylurea	Fluometuron	0.0×10^{-3}

the tested *N'*,*N'*-dimethyl phenylurea herbicides were hardly attacked by the enzyme system (Table 2).

Since the activity of the enzyme system responsible for inactivating *N'*-methoxy phenylureas was extremely low, only a 6.6-fold purification was achieved (Table 1). The enzyme system apparently hydrolyzed *N'*-methoxy phenylureas to the corresponding halogen-substituted anilines by two different processes. Enzymatic attack, resulting from a reaction with urease, would then form anilines, carbon dioxide from the ureido portion of the molecule, and a not yet defined compound derived from the *N'*-methoxy-*N'*-methylamine portion. The carbon dioxide production, however, is not related to the reaction with urease, since *B. sphaericus* does not possess this enzyme and none of the urea herbicides used in this investigation were degraded by soybean urease in vitro. These results may suggest that the cell-free system has amidase activity. Enzymatic attack at the amide linkage would yield a halogen-substituted phenylcarbamic acid and an unidentified metabolite. Phenylcarbamic acid is unstable and spontaneously disintegrates to the corresponding anilines and carbon dioxide (3).

It is difficult to explain why the decomposition appears to be specific for *N'*-methoxy phenylureas. The methoxy substitution may have a weakening effect on the bond between the ureido group and the substituted nitrogen because of the electrophilic nature of the oxygen (6). There is no indication that stepwise demethylation occurs before deamination takes place (1).

The highest enzymatic activity appeared in the late logarithmic-growth phase, before sporulation started (6). Therefore, a connection may exist between sporulation and appearance of the enzymatic activity in the hydrolyzation of *N'*-methoxy phenylureas.

ACKNOWLEDGMENT

This investigation was sponsored by the Federal German Ministry of Food, Agriculture, and Forestry.

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