

## Purification and Characterization of the *N*-Methylcarbamate Hydrolase from *Pseudomonas* Strain CRL-OK

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Received 9 July 1991/Accepted 27 September 1991

**A unique cytosolic enzyme that hydrolyzes the carbamate linkage of the insecticide carbaryl (1-naphthyl *N*-methylcarbamate) was purified from extracts of *Pseudomonas* sp. strain CRL-OK. Substrates of the hydrolase include the *N*-methylcarbamate pesticides carbofuran and aldicarb but not the phenylcarbamate isopropyl *m*-chlorocarbamate, the thiocarbamate *S*-ethyl *N,N*-dipropylthiocarbamate, or the dimethylcarbamate *o*-nitrophenyldimethylcarbamate.**

*N*-Methylcarbamate compounds such as carbaryl (1-naphthyl *N*-methylcarbamate) constitute a widely used class of pesticides which are generally not persistent in the environment (13). The lack of persistence of these compounds is due, in part, to the chemical instability of the carbamate bond, particularly under alkaline conditions (14). In addition, soil microbial enzymes such as bacterial carbamate hydrolases play a significant role in the biodegradation of these compounds (7, 11, 21). Indeed, with some soil-incorporated carbamate pesticides, microbial degradation processes may be so rapid so as to limit pesticide effectiveness in controlling targeted pests (1, 3, 9, 14, 18, 23, 24, 26, 29-31). At the other end of the spectrum, and in spite of their typically short half-lives, some carbamate compounds may be suspended from specific uses because of groundwater contamination (2).

A variety of microorganisms capable of transforming carbamate pesticides have been described in many studies (4-6, 10, 11, 16, 19, 22, 25, 27). However, there have been only a few biochemical studies of the enzymes responsible (7, 12, 15, 17). Our laboratory is particularly interested in carbamate hydrolase enzymes because of their potential role in the first step in the biodegradation of many carbamate compounds. In an effort to understand the diversity of degradation pathways in bacteria that utilize carbamate pesticides, we have isolated a group of bacterial strains that are capable of utilizing carbaryl as a sole source of carbon and energy. In this report we describe the isolation and partial purification of the *N*-methylcarbamate hydrolase from one isolate in this collection, *Pseudomonas* sp. strain CRL-OK.

Strain CRL-OK, a gram-negative rod, was isolated from sewage sludge from Oklahoma City, Okla. by selective enrichment with the insecticide carbaryl as the sole carbon and energy source. Taxonomic characterization of this strain was performed by using the Biolog GN-microplate system according to the instructions of the manufacturer (Biolog Inc. Hayward, Calif.) and an incubation time of 24 h at 30°C. On the basis of the Biolog plate results (data not shown), strain CRL-OK was assigned to the genus *Pseudomonas*. For purification of the *N*-methylcarbamate hydrolase, strain

OK was grown at 30°C in minimal medium (10) containing 20 mM sodium succinate as a carbon source and 5 mM methylaniline as a nitrogen source. The carbamate hydrolase was purified from the cytosolic fraction of cell extracts of strain OK by using DEAE, hydrophobic interaction, and gel filtration chromatography as described previously (12, 20). Our methods for performing size exclusion chromatography, sucrose density gradient centrifugation, protein determinations, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been described previously (12, 20).

Hydrolysis of carbaryl by crude and purified extracts was routinely measured by monitoring the appearance of the product of 1-naphthol and the sodium salt of the azo-dye Fast Blue RR (Sigma) at 600 nm. The molar extinction coefficient of this product was determined to be 14.6 M<sup>-1</sup> cm<sup>-1</sup> at 600 nm. Hydrolase assay mixtures (0.5 ml) contained 10 mM potassium phosphate (KPi) (pH 7.0), 0.08% SDS, 1.49 mM carbaryl (300 µg/ml), and 0.5 mg of Fast Blue RR salt, to which 5 µl of partially purified or purified hydrolase extracts was added. Because of spontaneous breakdown of the azo-dye, fresh 10-mg/ml stock solutions of this assay component (in 10 mM KPi [pH 7.0]) were prepared hourly. Carbaryl hydrolysis assays involving pH effects were performed by using Tris-HCl or KPi as pH buffers in separate experiments. Carbaryl hydrolysis assays involving temperature and 2-mercaptoethanol effects were monitored by high-performance liquid chromatography (HPLC) as described previously (7). Enzyme assays using the substrates *o*-NPDC (*o*-nitrophenyl-dimethylcarbamate), carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate), aldicarb [2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime], EPTC (*S*-ethyl *N,N*-dipropylthiocarbamate), and CIPC (isopropyl *m*-chlorocarbamate) were monitored by spectrophotometric and HPLC methods as previously described (7). The Michaelis-Menten constants of the hydrolase were determined by using carbaryl, carbofuran, and aldicarb concentrations between 30 and 152 µM, 28 and 141 µM, and 42 and 404 µM, respectively.

Hydrolase activity of crude extracts was relatively unstable at 4°C (with approximately 30% loss of activity after 24 h). However, the activity of crude extracts and of purified fractions was very stable during storage at -80°C (no detectable loss of activity after more than 1 month) and did not diminish after repeated cycles of freezing and thawing.

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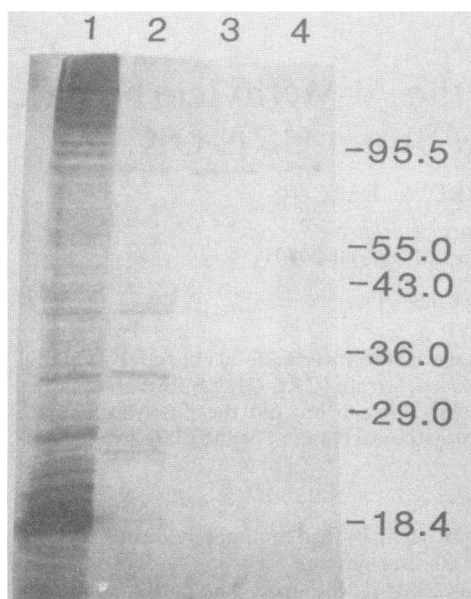


FIG. 1. Purification of the carbamate hydrolase from *Pseudomonas* strain CRL-OK. Samples were taken at different stages of hydrolase purification, subjected to SDS-PAGE, and silver stained. The molecular masses of protein standards (mid-range kit; Diversified Biotech, Newton Center, Mass.) are expressed in kilodaltons. Lanes: 1, crude soluble fraction; 2, pooled fractions after DEAE chromatography step; 3, pooled fractions after phenyl chromatography step; 4, pooled fractions after SW300 chromatography step. In the pooled SW300 fractions, several low-molecular-weight proteins are evident in addition to the 85,000-Da protein. Analysis of SW300 fractions showed that the presence of only the 85,000-Da protein correlated with carbamate hydrolase activity.

After partial purification by DEAE chromatography, hydrolase activity was very stable even at 4°C (no loss of activity after more than 1 month).

After ultracentrifugation of strain CRL-OK crude extracts, greater than 95% of the hydrolase activity was associated with the soluble fraction. Purification of the CRL-OK hydrolase to a single band of approximately 85,000 Da on SDS gels (Fig. 1) was accomplished by the chromatographic methods used (Table 1). The specific activity of the purified extracts increased nearly 1,100-fold during this procedure (Table 1). In repeated experiments, total hydrolase activity consistently increased after chromatography of the crude soluble fraction on the TSK-DEAE column.

The native molecular mass of the CRL-OK hydrolase was estimated by sucrose density gradient centrifugation to be

TABLE 1. Purification of carbamate hydrolase from *Pseudomonas* strain CRL-OK

Purification step	Total protein (mg)	Total activity (IU)	Sp act (IU/mg of protein)	Recovery (%)	Purification (fold)
Crude soluble	1,360	17.7	0.013	100	
TSK-DEAE	71.7	18.6	0.260	105	20
TSK-phenyl	2.75	7.88	2.86	44	220
300SW	0.016	0.23	14.1	— <sup>a</sup>	1,085

<sup>a</sup> Approximately 20% of the TSK-phenyl-purified material was subjected to 300SW chromatography. Of the portion which was run, the recovery of hydrolase activity was 39%.

	Substrate	K <sub>m</sub> (μM)
carbaryl		213
carbofuran		140
aldicarb		542
EPTC		ND*
o-NPDC		ND*
CIPC		ND*

\*Not determined. No activity detected.

FIG. 2. Michaelis-Menten kinetic constants (K<sub>m</sub>) for the carbamate hydrolase from *Pseudomonas* strain CRL-OK with carbamate substrates.

approximately 187,000 Da. This estimate is consistent with the subunit molecular size and suggests that the CRL-OK hydrolase is a dimer composed of two identical subunits of 85,000 Da.

To determine the substrate specificity of the CRL-OK hydrolase, Michaelis-Menten constants were estimated from least-squares regression of Woolf plots by using the methylcarbamates carbaryl, carbofuran, and aldicarb as substrates (Fig. 2). If the K<sub>m</sub> values are taken as a relative measure of the enzyme's affinity for these compounds, the CRL-OK hydrolase has approximately equal affinities for both carbaryl and carbofuran but a much lower affinity toward aldicarb. This enzyme displayed no detectable activity toward the carbamate compounds EPTC and CIPC or the colorimetric substrate o-NPDC.

Temperature and pH optima for the enzyme with carbaryl as substrate were determined by kinetic experiments. The pH optimum for the enzyme was between pH 8 and 8.5, and the temperature optimum was approximately 60°C.

Experiments designed to test the activity of the hydrolase in the presence of detergents, sulfhydryl reducing agents, and metal chelators demonstrated that the CRL-OK hydrolase retains activity over a wide range of reaction conditions. Most notable is the insensitivity of enzyme activity to the presence of the ionic detergent SDS. Hydrolase activity was slightly enhanced by low concentrations of SDS and retained greater than 70% of its activity in assay mixtures containing up to 0.5% SDS. In addition, hydrolase activity was not affected by the presence or absence of sulfhydryl reagents such as 2-mercaptoethanol (>90% activity in assays containing up to 0.2 mM 2-mercaptoethanol). Finally, the enzyme activity was unaffected in assays containing up to 10 mM EDTA and the activity was stable even after extended incubation (24 h, 4°C) of extracts in the presence of up to 10 mM EDTA.

Bacterial isolates displaying hydrolase activities specific for different classes of carbamate compounds have been described previously. Phenylcarbamate hydrolase activities have been characterized from two independently isolated *Pseudomonas* strains capable of utilizing the herbicide CIPC (15, 17). Although both enzymes were capable of hydrolyzing structurally related phenylcarbamates, neither displayed activity toward methylcarbamate compounds. *N*-Methylcarbamate hydrolase activities have been reported in cell extracts from two bacterial isolates, a *Pseudomonas* sp. (NCIB 12043) and *Achromobacter* strain WM111 (7, 12, 16). Of these two hydrolase activities, only the *Achromobacter* hydrolase has been isolated and characterized. While it possessed a broad substrate range among *N*-methylcarbamates, the *Achromobacter* hydrolase displayed no activity toward phenyl- or thiocarbamate compounds. The *mcd* gene, which is responsible for the *Achromobacter* hydrolase, has been recently isolated (28).

Characterization of the CRL-OK hydrolase revealed that it shares some features with the enzyme isolated from *Achromobacter* sp. strain WM111. Like the enzyme from the *Achromobacter* strain, the CRL-OK hydrolase is a cytosolic enzyme capable of hydrolyzing the methylcarbamate compounds carbaryl, carbofuran, and aldicarb and is composed of two subunits of identical size. However, the two enzymes differ with respect to the sizes of their subunits, their substrate ranges among methylcarbamate compounds, and their inhibition or stimulation by divalent cations and sulfhydryl reagents. Of particular note is the more limited substrate range of the CRL-OK hydrolase relative to the enzyme from the *Achromobacter* strain. Although both enzymes have roughly similar affinities toward carbofuran, only the *Achromobacter* enzyme is able to hydrolyze the dimethylcarbamate substrate *o*-NPDC. Indeed, none of the 36 isolates in our collection other than *Achromobacter* sp. strain WM111 displays detectable activity against *o*-NPDC (8). Whether this difference in substrate range is indicative of more fundamental differences in the active sites of these enzymes awaits more detailed study.

Microbial enzymes such as the *N*-methylcarbamate hydrolase of *Pseudomonas* sp. CRL-OK may play a significant role in "problem" or "aggressive" soils in which soil-incorporated pesticides are ineffective in controlling targeted pests. An understanding of these enzymes and of their diversity may therefore lead to an understanding of the problem soil phenomenon. To this end, continuing studies in

our laboratory are directed toward the isolation of the *Pseudomonas* CRL-OK hydrolase gene and toward characterization of other carbamate hydrolases.

We gratefully acknowledge Dan Shelton for isolating strain CRL-OK and Paul Tomasek and Janet Slovin for critically reading the manuscript.

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