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New Approaches to Identification and Activity Estimation of Glyphosate Degradation Enzymes

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Abstract—We propose a new set of approaches, which allow identifying the primary enzymes of glyphosate (N-phosphonomethyl-glycine) attack, measuring their activities, and quantitative analysis of glyphosate degradation *in vivo* and *in vitro*. Using the developed approach we show that glyphosate degradation can follow different pathways depending on physiological characteristics of metabolizing strains: in *Ochrobactrum anthropi* GPK3 the initial cleavage reaction is catalyzed by glyphosate-oxidoreductase with the formation of aminomethylphosphonic acid and glyoxylate, whereas *Achromobacter* sp. MPS12 utilize C-P lyase, forming sarcosine. The proposed methodology has several advantages as compared to others described in the literature.

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Phosphonates are organic compounds containing carbon-phosphorus (C–P) bonds in their structures, which makes them extremely resistant to acid and alkaline hydrolysis [1-3]. A number of biogenic phosphonates are known: 2-aminoethylphosphonate (2-AEP) isolated from flagellate protozoa from sheep rumen [2, 4], phosphonopyruvate, phosphonoacetate, and the antibiotic phosphonomycin (1,2-*cis*-epoxyprolylphosphonic acid) synthesized by species from the genus *Streptomyces*. Since the second half of the previous century synthetic phosphonates have begun to play an increasingly important role in human economic activities; they are used as lubricants, flame extinguishers, drugs, and biocides [4]. Among them, herbicides are currently leading in terms of application and in environmental pollution; glyphosate (N-phosphonomethyl-glycine, GP) is their acting component whose production is more than 500 thousand tons per year and increasing [5, 6].

Phosphonates are very stable, but microorganisms are known that can cleave the C–P bond in natural and synthetic phosphonates and use them as sources of phosphorus. Some enzymes of natural phosphonate synthesis and degradation have been identified, isolated, and studied and their metabolic pathways mapped [7]. However, the metabolism of synthetic phosphonates is still poorly understood. Only two enzymes have been found to primarily attack molecules of synthetic phosphonates: C-P lyase and glyphosate-oxidoreductase [4, 8].

C-P lyase breaks the C–P bonds in phosphonates of different structure, forming inorganic phosphate. Thus, P_i and sarcosine, later metabolized to glycine by sarcosine oxidase, are the products of the C-P lyase reaction in the case of GP [4, 9]. Attempts to study C-P lyase *in vitro* have not been successful because it is a complicated multienzyme complex and is irreversibly inactivated during the disintegration of cells [4, 10, 11].

Glyphosate-oxidoreductase catalyzes the cleavage of GP with the formation of glyoxylate and aminomethylphosphonic acid (AMPA). In contrast to C-P lyase, this enzyme retains its activity after the disintegration of cells. Its functioning has been estimated by accumulation of AMPA in the culture medium during GP consumption by bacteria at the beginning, and later by glyoxylate accumulation in homogenates in the presence of this compound [8, 12-14].

Lack of information about the enzymes of primary attack of phosphonates is largely due to the lack of reliable methods to identify and measure their activity. For example, an incorrect method of determining the activity of C-P lyase resulted in the mistaken "discovery" of this enzyme

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in cell-free extract of the bacterium *Enterobacter aerogenes* [15, 16]. NMR methods for identification of C-P lyase and GP-oxidoreductase metabolites containing isotopic labels described in the literature are highly accurate, but they require expensive equipment and their application for study of the dynamics of enzymatic reactions is difficult [17, 18]. Previously described approaches to the identification of phosphonates and their degradation products using high pressure liquid chromatography (HPLC) with fluorescence detection [19, 20], gas chromatography [21, 22], and thin layer chromatography (TLC) [23, 24] require specialized reagents and are time-consuming and unsuitable for studying the metabolism of these compounds. There are no rapid methods to detect and measure the activity of C-P lyase or GP-oxidoreductase.

GP was selected as a research object in the present study, the interest in which is explained by the urgency of the problem of large-scale soil and industrial waste contamination with GP-based herbicides, and by the need to develop methods for remediating the environment from this xenobiotic [25-27].

The goal of this study was to develop a set of methods to identify GP degradation enzymes and to estimate their activity.

MATERIALS AND METHODS

Reagents. We used the commercial product Roundup containing 320 g/liter of GP (Monsanto, USA); GP, AMPA, sarcosine, glyoxylate, glycine, sodium glutamate, 5-dimetilaminonaphtaline-1-sulfonyl chloride (dansyl chloride, DNS-Cl), FAD, and ammonium molybdate (Sigma, USA); H_2SO_4 (Merck, Germany); acetic anhydride, triethylamine, and isopropanol (Akron, Russia); 25% ammonia solution, 64% $HClO_4$, 36.5% HCl, ethyl acetate, chloroform, acetic acid, and sodium hydrocarbonate (Khimreaktiv, Russia); Tris, $MgCl_2 \cdot 6H_2O$ (Helicon, Russia); phenylhydrazine hydrochloride, benzoyl chloride, and EDTA·Na_2 (Fluka, Germany); DNase I (BioChemika, Germany); phenylmethylsulfonyl fluoride (PMSF) (Dia-M, Russia).

Microorganisms and their cultivation. We used two strains of bacteria that degrade phosphonates: *Ochrobactrum anthropi* GPK3, isolated from soils contaminated with GP, and *Achromobacter* sp. MPS12A, isolated from sites of contamination with methylphosphonic acid (MPA) and adapted to growth in GP-containing medium. The organisms were under periodic cultivation in liquid mineral medium MS1 without phosphates [28]. Sodium glutamate at concentration of 55 mM was used as a source of carbon. 3 mM GP as a Roundup component was used as a sole phosphorus source.

Acquisition of cell-free extract. Cells were collected at logarithmic growth phase, centrifuged for 30 min at 5000*g*, and the pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.65. Centrifugation was repeated, the cells were resuspended in the same buffer with 75 mM NaCl, 0.05 mM EDTA, 0.01 mM PMSF, and 20% (v/v) glycerol and precipitated again. The precipitate was frozen at −70°C and subjected to extrusion with a Hughes press at working pressure of 0.9 MPa. One hundred units of DNase I were added to the resulting homogenate. Sarcosine oxidase inhibitor (sodium acetate at final concentration of 10 mM) was added in the experiments on sarcosine detection. The homogenate was then centrifuged at 4°C for 40 min at 30,000*g*. Supernatant, containing proteins of cytoplasm fraction, was filtered through a Whatman GD/X membrane filter (Whatman, USA) with pore diameter of 0.2 μm.

Protein concentration was determined by the Bradford method.

Identification of GP and its degradation products using chromatographic methods. Samples were derivatized to reduce the detection limits of compounds.

Sample preparation for N-acyl derivatives. Ten microliters of acetic anhydride and 1 μl of triethylamine were added to a 100 μl sample, and the tube was tightly closed and kept at room temperature for 30 min. The resulting mixture was placed in a vacuum desiccator containing alkali to remove the byproduct of acylation – acetic acid. The precipitate was dissolved in 5 mM H_2SO_4 before analysis [29].

Sample preparation for N-dansyl derivatives. Samples were dansylated at pH 9.0-9.5; a 90 μl sample was mixed with 10 μl 1 M $NaHCO_3$ and 100 μl of 37 mM dansyl chloride solution in acetone, then the mixture was incubated overnight at room temperature in the dark [30].

Sample preparation for N-benzoyl derivatives. One hundred microliters of sample was added to 2 ml of 1.5 M $NaHCO_3$ solution, 150 μl of benzoyl chloride was added, and the tube was vortexed for 90 min. The mixture was filtered through a DynaGard-ME membrane filter (Spectrum Laboratories, USA) with pore diameter of 0.2 μm, acidified with 250 μl of 1 M HCl, and then used for analysis.

Analysis of GP and its metabolites with HPLC. To analyze the decline of GP content and the accumulation of its metabolites, we prepared a series of mixtures of 0.8 ml volume containing 0.1 M Tris-HCl, pH 8.0, 10 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM FAD, 10 mM GP, and 0.2 ml of cell-free extract.

The mixture was vortexed and incubated at 30°C after addition of all the components. The reaction was stopped with an interval of 5 min by addition of 0.2 ml 64% perchloric acid. Denatured proteins were precipitated by centrifugation at 15,000*g* for 10 min, the supernatant was filtered through a DynaGard-ME membrane filter, and the filtrate was derivatized according to the methods described above and analyzed by HPLC. A sample in which perchloric acid was added immediately after mixing was used as control.

Studies were performed using an LKB-Bromma 2150 liquid chromatograph (LKB, Sweden) with UV detector with operating wavelength of 210 nm for acyl, 225 nm for benzoyl, and 330 nm for dansyl derivatives. We used a Repro-Gel H 250 × 8 mm column, at a temperature of 65°C (Dr. Maisch, Germany) and 5 mM H_2SO_4 as mobile phase with flow rate of 1 ml/min for analysis of acyl and benzoyl derivatives. We used a ReproSil-PAH EPA column (Dr. Maisch), at a temperature of 45°C and a mixture of methanol–20 mM CH_3COONa, pH 5.1 (20 : 80 v/v) as mobile phase for analysis of dansyl derivatives.

Analysis of GP and its metabolites using TLC. Chromatographic mobility of GP and its microbial degradation products was determined on Sorbfil plates PTSH-P-V (Sorbpolimer, Russia) in ascending manner in a glass chamber with mobile phase of isopropanol–5% ammonia (1 : 1 v/v). The plate was dried for 10 min in a stream of air at room temperature after chromatographic separation. The plate was sprayed with 0.25% (w/w) solution of ninhydrin in acetone and heated for 1-2 min at 80°C for detection of amines. We used 80 mM solution of ammonium molybdate in a mixture of H_2O–HCl (1 : 1 v/v) as an indicator for phosphorus, which gave white spots on blue background.

Dansyl phosphonic acids derivatives were separated by one-dimensional chromatography with sequential use of two mobile phases. First, the plate was chromatographed in chloroform–methanol–acetic acid (25 : 5 : 0.2 v/v) for separation of DNS-OH, DNS-Cl, and dansyl amines present in the reaction mixture. After drying of the silica gel in an air flow, the plate was re-chromatographed in the main system of ethanol–24% ammonia solution (7 : 3 v/v). Sarcosine was identified by two-dimensional TLC, for which the plate was chromatographed with ethyl acetate–isopropanol–24% ammonia (45 : 35 : 20 v/v) mobile phase, and then with chloroform–methanol–acetic acid (25 : 5 : 1 v/v) in the perpendicular direction. The presence of glycine was confirmed by one-dimensional chromatography of the plate in the same system. The compounds were detected using a UFS 254/365 Chromatographic Irradiator (Sorbpolimer) at wavelength of 365 nm.

R_f values were calculated based on the results of three tests.

Spectrophotometric determination of GP-oxidoreductase activity in cell-free extract. The method is based on the ability of glyoxylate, formed by cleavage of GP, to react with phenylhydrazine producing hydrazone with a maximum optical absorption at 324 nm. The reaction mixture with total volume of 3 ml containing 0.1 M Tris-HCl, pH 8.0, 10 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM FAD, 2 mM phenylhydrazine hydrochloride, 2 mM GP, and 2 ml of cell-free extract was used for the assay. The measurement was performed at 30°C for 2 min on UV-1650PC spectrophotometer (Shimadzu, Japan). A cell with a similar reaction mixture containing no GP was used as a reference. Activity was calculated by increase in A_{324} using glyoxylate phenylhydrazone molar extinction coefficient of $1.7 \cdot 10^4\ M^{-1} \cdot cm^{-1}$, and expressed in micromoles of glyoxylate in 1 min per mg protein.

RESULTS

HPLC of GP and its metabolites. Identification of products of glyphosate degradation by GP-oxidoreductase (AMPA and glyoxylate) and C-P lyase (sarcosine) with UV detection is impossible because these compounds poorly absorb light at the operating wavelength of 210 nm, and it is practically impossible to detect them under these conditions. For the analysis of the compounds we used their acyl, benzoyl, and dansyl derivatives.

Table 1. Analysis of GP derivatives and metabolites by HPLC

Compound	Acyl derivatives		Benzoyl derivatives		Dansyl derivatives	
	Retention time, min	Detection limit, ng/μl	Retention time, min	Detection limit, ng/μl	Retention time, min	Detection limit, ng/μl
GP	19.97	0.60	43.16	0.36	4.73	0.16
AMPA	4.98	0.90	6.52	0.44	13.31	0.48
Sarcosine	11.00	0.86	21.44	0.74	26.16	0.33
Glycine	9.20	0.40	19.73	0.36	14.67	0.29
Glyoxylate	5.40	0.76	5.40	0.76	n.d.	n.d.

Note: n.d., not detected.

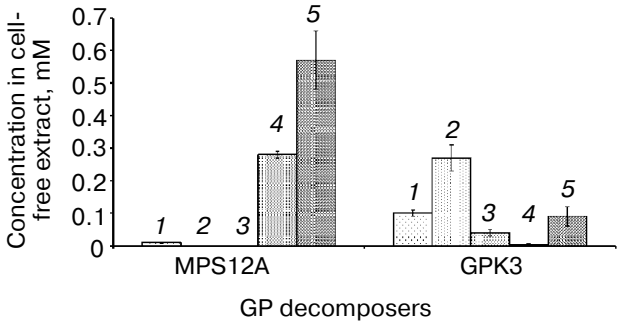

Fig. 1. Concentrations of acyl derivatives of GP (*1*) and its metabolites AMPA (*2*), glyoxylate (*3*), sarcosine (*4*), and glycine (*5*) in cell-free extracts of GP-metabolizing strains *Achromobacter* sp. MPS12A and *O. anthropi* GPK3. Protein content in homogenates in all experiments was 7-8 mg/ml.

Acyl and benzoyl derivatives separated out well in the strong anion exchanger Repro-Gel H in the liquid phase of 5 mM H_2SO_4 and eluted in the order shown in Table 1.

The best separation of dansyl derivatives of GP and its metabolites was observed while eluting from the column Repro-Sil PAH EPA with mixture of methanol and 20 mM acetate buffer, pH 5.1, as the mobile phase. Parameters of separation of the compounds were determined by the concentration of methanol in the mobile phase: elution time of all dansyl derivatives decreased with increasing concentration. Volume ratio 20 : 80 of methanol and acetate buffer provides the greatest resolving ability of the system. Conditions of dansyl derivative elution are given in Table 1.

The chosen conditions of analysis allowed not only separation and identification, but also quantification of the acyl, benzoyl, and dansyl derivatives of the studied compounds. Calculations were performed based on calibration curves of chromatogram peak area dependence on concentration of substance in the standard solution.

Analysis of acyl derivatives showed that only sarcosine and glycine were present in cell-free extract of strain *Achromobacter* sp. MPS12A, whereas AMPA and glyoxylate and only trace amounts of sarcosine and glycine were detected in homogenate of *O. anthropi* GPK3 (Fig. 1). Similar results were obtained for dansyl and benzoyl derivatives (data not shown).

TLC of GP and its metabolites. The developed TLC method reliably separated GP, AMPA, sarcosine, and glycine in culture broth and cell-free extracts of cells both in unmodified form and as dansyl derivatives. It confirmed the presence of sarcosine and glycine in cell-free extracts of strain MPS12A and the presence of AMPA in homogenates of strain GPK3. The R_f values for each of these compounds agreed with the R_f of standard samples. Data on their mobility are given in Table 2.

The described methods for analysis of GP and its metabolites were used for the detection of C-P lyase and GP-oxidoreductase in bacterial strains *Achromobacter* sp. MPS12A and *O. anthropi* GPK3. We did not detect the presence of AMPA and glyoxylate in cell-free extract of MPS12A strain, but identified sarcosine and glycine there (Fig. 1). In contrast, AMPA, glyoxylate, and only trace amounts of sarcosine and glycine were found in cell-free extract of GPK3 (Fig. 1).

Spectrophotometric estimation of GP-oxidoreductase activity. Considering that glyoxylate is one of the products of GP cleavage by this enzyme, we developed a spectrophotometric method for determining its activity in cell-free extract of strain GPK3 based on the rate of glyoxylate hydrazone formation. Since the formation of hydrazones may occur in the reaction not only with glyoxylate, but also with a number of other cellular metabolites with aldo- or keto-group, a nonspecific increase in A_{324} value was observed for some time upon introduction of cell-free extract into the measuring cell. After reaching of a plateau and introduction of GP into the cuvette, the increase in absorbance was linear, caused by glyoxylate formation in enzymatic GP cleavage (Fig. 2). It was found that nonspecific increase in A_{324} in the initial period of measurement did not occur when using the reaction mixture with cell-free extract, but without GP, as a refer-

Table 2. Chromatographic mobility and detection limits of GP and its metabolites identified by TLC in unmodified state with ninhydrin and for dansyl derivatives

Compound	No modifications		Dansyl derivative	
	R_f, %	Detection limit, ng	R_f, %	Detection limit, ng
GP	33 ± 1	600	32 ± 1	30
AMPA	25 ± 1	500	49 ± 1	35
Sarcosine	54 ± 2	250	–*	1
Glycine	64 ± 2	200	23 ± 1	1

* Sarcosine was detected by two-dimensional TLC.

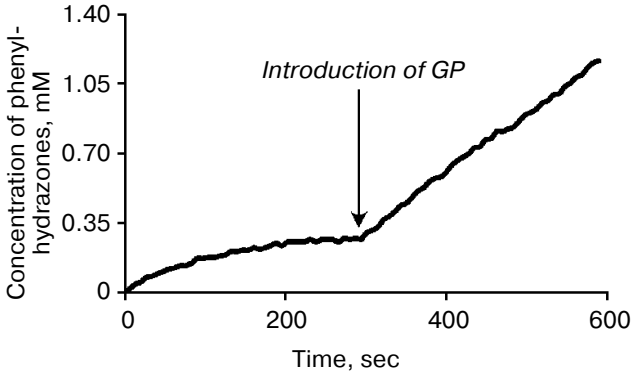

Fig. 2. Spectrophotometric measurement of phenylhydrazone concentration in reaction mixture containing GP and cell-free extract of *O. anthropi* GPK3.

ence, which made it possible to apply this technique for measuring the enzyme activity.

The specific activity of GP-oxidoreductase measured spectrophotometrically was 0.033 ± 0.003, and when measured with HPLC it was 0.034 ± 0.002 μmol GP for 1 min per mg protein.

DISCUSSION

Due to the lack of direct methods for determining the activity of GP degradation enzymes, chromatographic methods of analysis were used to estimate the degradation of substrate and the formation of its metabolites in the culture medium and cell homogenates of metabolizing strains. Taking into account specificity of the studied objects, which could contain a variety of intracellular metabolites, we have proposed new approaches within the methods of TLC and HPLC allowing quick identification of GP and products of its utilization and to reveal the degradation pathways of this phosphonate.

Identification of GP and its metabolites in unmodified form by TLC with development of plates with ninhydrin is complicated by the presence of biogenic amines in cell homogenates of GP-degrading strains. In this case, GP and AMPA could be selectively identified with ammonium molybdate treatment. However, analysis sensitivity for unmodified compounds during development with either ninhydrin or ammonium molybdate is inadequate. Use of dansyl derivatives of GP and its metabolites for analysis reduced the detection limit of these compounds while maintaining the reliability of GP degradation product detection in complex mixtures. The disadvantage of this method was the impossibility of simultaneously separating all investigated metabolites in the same solvent system.

The TLC method can be used for rapid preliminary identification of these compounds in culture media and in reaction mixtures containing cell-free extracts of GP metabolizing strains.

The impossibility to use HPLC with UV detection for the analysis of the compounds in unmodified form resulted in the necessity to obtain the acyl, benzoyl, and dansyl derivatives.

Among HPLC-based methods of analysis devised by us, analysis of acyl derivatives and metabolites of GP is the least time consuming. However, analysis of cell-free extract with this method is difficult due to the large number of compounds present in the sample that elute near the peaks of acyl-AMPA and acyl-glycine.

HPLC of benzoylated samples allows better separation of AMPA, sarcosine, and glycine derivatives from the other cytoplasmic components, and also somewhat lowers detection limit of the tested compounds. However, the separation of AMPA/GP/sarcosine/glyoxylate mixture in benzoylated form is twice as long as for acyl derivatives. In addition, the elution time of some cytoplasmic components increases several-fold after benzoylation, which requires flushing of the chromatographic column with liquid phase for 40 min after each separation.

Dansylation of samples provided the lowest detection limits of the investigated compounds and their good separation even in complex mixtures such as cell-free extract, with the exception of the dansyl glycine/dansyl AMPA couple. It can be noted as a disadvantage of this method that the time required for dansylation of samples is relatively long.

In this paper, acyl derivatives analysis was used most commonly as the fastest and most convenient method. Detection reliability for AMPA and glycine was monitored with benzoyl derivatives, and dansylation of samples was performed to identify trace amounts of sarcosine and GP.

The HPLC method not only allowed qualitative analysis of cell-free extracts for detection of GP metabolites with high sensitivity, but it also allowed estimation of the concentration change for both substrate and products of GP degradation by various enzyme systems, which is especially important in determination of their activity in cell-free extract containing many cellular metabolites adversely affecting the accuracy of measurements. An important condition for the analysis using cell-free extract was reaction arrest and protein denaturation by the reaction mixture with perchloric acid. This technique allows more accurate determination of GP concentration as compared with boiling, where a considerable error in measurement is observed, apparently due to sorption of GP by cytoplasmic components (data not shown). The presence of perchloric acid did not lead to decomposition of GP present in a sample for at least three days at room temperature.

We suggest the spectrophotometric method of measuring GP-oxidoreductase activity by reaction of formed glyoxylate with phenylhydrazine as a replacement for the

existing method [8, 14] based on a similar reaction of glyoxylate with 2,4-dinitrophenylhydrazine, but requiring an additional HPLC phase for the separation of glyoxylate hydrazone from other hydrazones of aldo- and keto-compounds present in cell-free extract. Our use of a cuvette containing cell-free extract as a control removed nonspecific increase in value of A_{324}, which significantly reduced the time required for one analysis and made it possible to observe the reaction dynamics in real time. GP-oxidoreductase activity values, obtained by spectrophotometric measurement, were comparable with data of quantitative HPLC of GP decrease in reaction mixture containing cell-free extract. Therefore, this rapid method of measuring the activity of GP-oxidoreductase is reliable.

Detection of sarcosine after addition of a specific sarcosine oxidase inhibitor, sodium acetate, to the cell-free extract of *Achromobacter* sp. strain MPS12A, indicates the participation of C-P lyase in the metabolism of this phosphonate. Subsequent transformation of sarcosine results in glycine, the increased content of which was observed in cells of strain MPS12A compared to strain GPK3 (Fig. 1). The presence of AMPA and glyoxylate in cells of *O. anthropi* strain GPK3 indicated other paths of GP utilization catalyzed by GP-oxidoreductase. At the same time, the cytoplasm of GPK3 as well was shown to contain sarcosine, but at concentrations 3-4 orders of magnitude lower compared to the abovementioned metabolites; therefore, C-P lyase does not play an important role in the utilization of GP in this strain.

Thus, proposed methods not only allow reliable identification and measurement of the activity of enzymes of GP metabolism, but also give important information about the diversity of GP metabolic pathways in soil bacteria.

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