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Enzymic Degradation of Bromoxynil by Cell-Free Extracts of *Streptomyces felleus**

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ABSTRACT. A method is described for spectrophotometric monitoring the degradation of the herbicide bromoxynil by cell-free extracts of *Streptomyces felleus*. The method involves a decrease in absorbance at 286 nm (absorption maximum of bromoxynil) that can be ascribed most probably to the cleavage of the aromatic ring of the bromoxynil molecule. Conditions necessary for measuring this degradation together with physico-chemical features of the degradation indicate that the reaction(s) is seemingly catalyzed by an Fe^{2+}-dependent dioxygenase whose activity was not, however, detected in cell-free extracts of a bromoxynil-sensitive mutant of *S. felleus* as well as other bromoxynil-sensitive streptomycete strains.

Bromyxynil (3,5-dibromo-4-hydroxybenzonitrile) is a contact herbicide effective against dicotyledonous weeds (Carpenter and Heywood 1983). A streptomycete strain was found that was not only able to grow in the presence of bromoxynil (BX) in a medium but also supported growth and development of BX-sensitive streptomycetes in a BX-containing medium (Krištůfek and Blumauerová 1983*a,b*).

The BX-resistant strain was determined by the ISP method (Shirling and Gottlieb 1966) as *Streptomyces felleus* (Krištůfek *et al.* 1987*a*). Degradation of BX by *S. felleus* was brought about by cometabolism in the presence of a utilizable source of nutrients and energy.

It is well established that numerous aromatic pesticides are degraded by soil microorganisms by way of cleavage of the aromatic ring catalyzed by various dioxygenases (Bollag 1974). Accordingly, previous results indicate that the degradation of BX by *S. felleus* could be due to the fission of the benzene ring of the molecule (Krištůfek *et al.* 1987*a*).

This led us to search for an enzymic activity responsible for the degradation of the BX molecule.

*Part 3 of the series Resistance against the herbicide bromoxynil in *Streptomyces felleus*. Part 2: *Folia Microbiol.* 32, 305–313 (1987).

MATERIAL AND METHODS

Chemicals. Bromoxynil was purchased from *Lachema* (Czechoslovakia), NADP and phenylmethanesulfonyl fluoride was from *Serva* (FRG), NADH and ATP from *Reanal* (Hungary), NAD from *Merck* (FRG), NADPH from *Fluka* (Switzerland), glutathione from *Koch-Light* (United Kingdom) and Sephadex G-25 (medium) from *Pharmacia* (Sweden). All other chemicals were of the highest purity commercially available.

Microorganisms and cultivation conditions. The following microorganisms were used: *Streptomyces felleus* P-14, a wild strain resistant to BX (Krištůfek and Blumauerová 1983*a*); *S. felleus* P-14/2, an acriflavine-induced mutant of *S. felleus* P-14 sensitive to BX (Krištůfek *el al.* 1983*b*); *S. aureofaciens* 84/25 (Research Institute of Antibiotics and Biotransformations, Roztoky near Prague); *S. erythreus* 1815 and *S. fradiae* B/18 (Institute of Microbiology, Czechoslovak Academy of Sciences, Prague).

Microorganisms were cultivated under shake-flask conditions (reciprocal shaker, 1.6 Hz) at 28 °C in 300-mL Erlenmeyer flasks, each containing 50 mL yeast extract-malt extract medium comprising (g/L): yeast extract 4, malt extract 10, glucose 4 (pH 7.3). The cultures were inoculated with 5 mL of a vegetative mycelium grown under the above conditions for 24 h.

Preparation of cell-free extracts. The mycelium of a strain was separated from the fermentation broth by two centrifugations (Janetzki K26, 4000 ***g***, 15 min), washed with ice-cold water, centrifuged (Sorvall, 20 000 ***g***, 30 min) and kept frozen.

The cells were disintegrated in a bacterial X-press (Biox AB, −25 °C, 20−30 MPa), homogenized in a glass-piston homogenizer (Kavalier) in a buffer containing Tris-HCl (100 mmol/L, pH 7.4), EDTA (2 mmol/L), 15 % (*V*/*V*) glycerol, 2-mercaptoethanol (1 mmol/L) and phenylmethanesulfonyl fluoride (0.1 mmol/L) and centrifuged (Sorvall, 20 000 ***g***, 30 min). The resulting supernatant was desalted using a Sephadex G-25 column and applied immediately as an enzyme preparation.

Determination of protein. The protein concentration was measured by Lowry's method using bovine serum albumin as standard.

Assay of BX-cleavage activity. The activity was measured as a decrease in absorbance at 286 nm using a Cary 118 C spectrophotometer (Varian) in an assay mixture comprising Tris-HCl (50 mmol/L, pH 7.4), $FeSO_4$ (1 mmol/L), BX (7 μmol/L), plus 5 μL cell-free extract (1 μg protein) of *S. felleus* P-14 in a total volume of 1 mL. Assays were performed at 28 °C after a 3-min preincubation (baseline adjustment). The reaction was started by the addition of BX. The calculation of the activity was based on the molar absorptivity of BX being 1.93×10^3 m^2/mol and specific activities were expressed in μkat per mg protein.

A control experiment was done to exclude nonenzymic cleavage of BX. In this case the reactions were run in the absence of the *S. felleus* P-14 cell-free extract.

Assay of catechol 1,2-dioxygenase activity. Pyrocatechol 1,2-dioxygenase (EC 1.13.11.1) was measured as an increase in absorbance at 260 nm in an assay mixture containing in a total volume of 1 mL pyrocatechol (0.2 mmol/L), phosphate buffer (0.1 mol/L, pH 7.5) plus an appropriate amount of cell-free extract of *S. felleus* P-14 (Hayaishi *et al.* 1957).

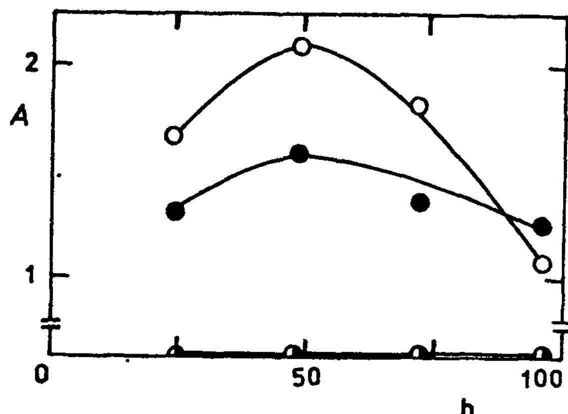

FIG. 1. Time course (h) of BX-cleavage activity (specific activity A, μkat/mg) in cell-free extracts of *S. felleus* P-14 in the presence (*open symbols*) and absence (*closed symbols*) of BX in the cultivation medium and in cell-free extracts of *S. aureofaciens* 84/25 (*half-closed symbols*).

RESULTS

Determination of BX-cleavage activity and its properties

The activity was determined at 286 nm monitoring the decrease in absorbance, the chosen wavelength corresponding to the absorption maximum of BX.

When the cell-free extract of *S. felleus* P-14 was omitted in the assay mixture, the decrease in absorbance at 286 nm was not detected. Thus a non-enzymic cleavage of BX can be excluded.

A number of low-molar-mass effectors and cations were tested for their possible effect on the BX-cleavage activity in 1 mmol/L concentration. These include NAD^+, $NADP^+$, NADPH, ATP, glutathione (reduced), Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{3+} and Fe^{2+} and ATP, $NADP^+$ and NADPH in combination with Mg^{2+}. Interestingly, the activity was detected only in the presence of Fe^{2+} ions and these could not be replaced with any of the cations tested. Furthermore, the activity was not detected when cofactors, such as NAD^+, $NADP^+$, NADPH, glutathione (reduced) or ATP, were added to the reaction mixture, the Fe^{2+} cations being omitted.

The pH optimum turned out to be at 7.4 and the enzyme was active only in a very narrow pH range. Similarly, the activity dropped considerably with even a slight temperature rise as well as with storage (*data not shown*) so that fresh enzyme preparations had to be used.

When the cell-free extract of *S. felleus* P-14 was incubated with catechol, no corresponding activity of pyrocatechol 1,2-dioxygenase was detected.

Among several streptomycete strains (*S. felleus* P-14, *S. felleus* P-14/2, *S. aureofaciens* 84/25, *S. erythreus* 1815, *S. fradiae* B/18) BX-cleavage activity was detected only in the cell-free extract of the BX-resistant strain *S. felleus* P-14. Absence of the activity in other streptomycete strains under study was confirmed by an experiment in which a time course of the activity was followed. The activity (Fig. 1) did not exhibit itself in any phase of cultivation of *S. aureofaciens* 84/25, a highly BX-sensitive strain (the lethal dose being 20 μg BX per mL cultivation medium).

Inducibility of BX-cleavage activity. In order to elucidate whether the BX-cleavage activity of *S. felleus* P-14 cell-free extract is inducible, BX was

added to the cultivation medium at an appropriate concentration (50 μg BX per mL medium) that did not interfere with the growth of the strain *S. felleus* P-14.

It was revealed that the activity in extracts of mycelium grown in the presence of BX was 1.5-times higher than that in extracts grown in the absence of BX. The maximum activity was found in 2-d-old cultures grown both with and without BX in the cultivation medium (Fig. 1).

DISCUSSION

Many herbicides are aromatic compounds whose biodegradation can only occur after cleavage of the benzene ring. Various soil microorganisms have the ability to oxidize aromatic substrates and utilize the resulting aliphatic compounds as substrates in their intermediary metabolism (Bollag 1974). Accordingly, the biodegradation of the herbicide bromoxynil was reported for both *in vivo* (Smith 1971, Krištůfek *et al.* 1988) and *in vitro* conditions (Smith and Cullimor 1974, Krištůfek *et al.* 1987*b*, McBride *et al.* 1986).

The ability of the cell-free extract of *S. felleus* P-14 to cleave the benzene ring of BX could be conveniently detected spectrometrically. The decrease in absorbance at 286 nm (absorption maximum of BX) after incubation with the *S. felleus* P-14 cell-free extract agrees which earlier results where the absorption band at 286 nm in UV spectra of extracts from solid media supplemented with BX and inoculated with *S. felleus* P-14 spores disappeared upon cultivation; likewise, the peak corresponding to the BX standard vanished when these extracts were subjected to reversed-phase high-performance liquid chromatography (Krištůfek *et al.* 1987*a*).

Of a number of cofactors and cations that are often essential in reactions catalyzed by dioxygenase, only Fe^{2+} ions were functional. Moreover, the BX-cleavage activity was not seen in the absence of Fe^{2+} ions or when divalent iron was replaced with other divalent ions or Fe^{3+} ions. Similarly, the BX-cleavage activity was strictly pH-dependent, a slight change of the pH optimum bringing about a dramatic reduction of the activity (the activity could be detected in a pH range of about 7.2 to 7.6). It may be that a shift of pH causes oxidation of Fe^{2+} to Fe^{3+} which in some cases inhibits the Fe^{2+}-dependent dioxygenases.

To elucidate whether the resistance to BX brought about by the degradation of this toxic compound was present exclusively in the strain *S. felleus* P-14, a representative selection of streptomycete strains was done.

Mutagenic treatment of the original strain *S. felleus* P-14 with acriflavin resulting in the loss of resistance to BX (strain *S. felleus* P-14/2; Krištůfek and Blumauerová 1983*b*) also eliminated the BX-cleavage activity. As expected, the activity was not detected in the highly BX-sensitive strain *S. aureofaciens* 84/25. Lethal doses for these two sensitive strains were 20 μg BX per mL fermentation medium while the BX-resistant strain *S. felleus* P-14 could grew at 400 μg BX per mL cultivation medium (Krištůfek and Blumauerová 1983*a*).

As *S. felleus* is a producer of pikromycin, a 14-membered macrolide antibiotic, two other macrolide producers, *S. erythreus* 1815 and *S. fradiae* B/18, were included so that similar metabolic processes involved in these strains might be reflected in the properties of corresponding cell-free extracts in terms of BX-cleavage activity. Moreover, *S. fradiae* was recently reported to

possess dioxygenase activities participating in the biosynthesis of tylosin, its macrolide metabolite (Omura *et al.* 1984). However, BX-cleavage activity was detected in none of these strains emphasizing its occurrence only in *S. felleus* P-14.

The BX-cleavage activity turned out to be present in the cells on a basal level that was increased approximately 1.5-fold upon addition of its substrate, BX, to the cultivation medium. This feature is in accord with other microbial strains that cannot utilize pesticides as sole sources of carbon and energy and these are cometabolized and, furthermore, the levels of enzymes responsible for their degradation are enhanced upon their addition to the cultivation media (Jensen 1960).

The question remains unanswered what the products are of the reaction(s) catalyzed by the enzyme activity reported here. No products of degradation of BX by *S. felleus* P-14 containing an aromatic ring were detected (Krištůfek *et al.* 1987*b*). We suppose that in *S. felleus* P-14 BX is degraded by aromatic ring cleavage resulting in aliphatic compound(s) that, presumably after modification of the nitrile group and debromination, immediately enter the intermediary metabolism of the cell. In contrast, a strain of *Flexibacterium* sp. BR4 degraded BX by nitrile group modification and debromination resulting in several compounds possessing an intact aromatic ring (Smith and Cullimor 1974). Recently, a strain of *Klebsiella pneumoniae* subsp. *ozaenae* was reported to metabolize BX by nitrile group modification that is mediated by a highly BX-specific nitrilase (EC 3.5.5.-) liberating ammonia from the BX molecule (serving as a sole nitrogen source for the bacterium) and yielding 3,5-dibromo-4-hydroxybenzoic acid (a nonmetabolizable end product in this strain) that is transported outside the cell (McBride *et al.* 1986).

A number of streptomycete strains were reported to exhibit dioxygenase activities that catalyze general reactions involved in intermediary metabolism, *e.g*, catabolite reactions of aromatic substrates (Antai and Crawford 1983, Pometto *et al.* 1981, Pometto and Crawford 1985, Sutherland 1986). Contrary to these findings, it is likely that "general" dioxygenase activities are not so widespread in *S. felleus* P-14 as indicated by the absence of pyrocatechol 1,2-dioxygenase that was chosen as a "marker" enzyme of common ring-cleaving dioxygenases.

The results presented in this communication together with the previous ones (Krištůfek *et al.*, 1987*a*,*b*) suggest that we are probably dealing with a new dioxygenase, its substrate being a rather unusual and toxic compound whose activity was not detected in a number of other streptomycete strains. Moreover, this activity seems to be very sensitive to pH and temperature changes necessitating the assay conditions to be carefully balanced and impeding work with this activity. Our further attempt should be to purify this activity and study its properties as the strain *S. felleus* P-14 represents an example how some microorganisms "cope with" toxic compounds that sometimes remain in soil and utilize them in their metabolism and involves promising environmental implications.

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